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Sex-specific Modulation of Drug Bioavailability with Pharmaceutical Excipients

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A thesis submitted in partial fulfillment of the requirements of UCL School of Pharmacy for the degree of Doctor of Philosophy

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Declaration

This thesis describes the research conducted in the School of Pharmacy, University College London between 2013 and 2017 under the supervision of Professor Abdul Basit. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publications.

Signature: _____ Date: _____

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Abstract

Pharmaceutical excipients are usually considered as “inert” ingredients according to pharmaceutical regulations and standards. The solubilizing excipient polyethylene glycol 400 (PEG 400), however, unexpectedly alters the oral bioavailability of the BCS class III drug, ranitidine, in a sex-dependent manner. A 63% increase in bioavailability was exhibited in male rats, however, no increase was identified in female counterparts. This finding has significant implications for the use of supposedly “inactive” excipients and additionally highlight distinct differences, simply due to the sex of the animal. The aim of this thesis was to clarify the mechanism underlying this sex-associated phenomenon, alongside the discovery of a variety of “active” excipients that potentially exhibit sex-related behavior. The work involved an assessment on the influence of PEG 400 on the bioavailability of a number of BCS class III drugs in male and female rats. The presence of PEG 400 only showed sex-based enhancement on the absorption of drugs (ranitidine and ampicillin) which are substrates for efflux transporter, P-glycoprotein (P-gp). A step-by-step investigation then revealed that PEG 400 was capable of decreasing the overall P-gp activity, protein abundance and mRNA expression. This, however, was only observed in male rats and not in females. Besides PEG 400, a number of pharmaceutical excipients including Cremophor RH 40, Poloxamer 188 and Tween 80 also demonstrated a sex-specific reduction on the function of P-gp to increase the bioavailability of P-gp-mediated drugs. Overall, the results are promising and demonstrate that excipients and sex of the organism have a major influence on drug bioavailability. The results of the investigation contributed to establish a small database of excipients, commonly selected in oral formulation design that researchers should acknowledge and avoid to ensure therapeutic efficacy and to limit side effects between males and females.

Impact Statement

The biopharmaceutics classification system (BCS) is a framework for classifying drug substances based on their aqueous solubility and permeability across a biological membrane. It is possible for individuals to request a waiver of *in vivo* bioequivalence (BE) studies for immediate-release solid oral dosage forms. Nowadays, the Food and Drug Administration allows the biowaiver for BCS Class I and Class III drugs. However, the findings in this thesis call attention to the unearthed loopholes in this regulation, given that formerly considered "inert" pharmaceutical excipients have shown unexpected impacts on the absorption of co-formulated drugs. By virtue of our studies, it has been exposed that a number of excipients used to aid tablet manufacturing or formulation development are able to enhance therapeutic efficacy of the drug by the modulation of transporter activity and expression. This, therefore, could characterize the excipient to have two applications; in manufacturing and drug formulation. Furthermore, the thesis outlines the modifications of the overall function of transporters, simply due to the sex of the organism. As a result, the work reported here emphasizes that the selection of excipients during formulation development is integral in preventing differences in therapeutic efficacy and side effect profiles between males and females.

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List of Abbreviations

SLC	The solute carrier
ABC	ATP-binding cassette transporters
OATP	Organic anion transporting polypeptide
OAT	Organic anion transporter
OCT	Organic cation transporter
OCTN	Organic carnitine transporter
CNT	Concentrative nucleoside transporter
ENT	Equilibrative nucleoside transporter
MATE	Multidrug and toxin extrusion transporter
MDR	Multidrug resistance protein
MRP	Multidrug resistance-associated protein
BCRP	Breast cancer resistance protein
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
MXR	Mitoxantrone resistance
NTCP	Sodium-dependent taurocholate uptake transporter
TEA	Tetraethylammonium
BLM	Basolateral membrane
OGTT	Oral glucose tolerance test
CYPs	Cytochromes P450
V_p/F	Peripheral volume of distribution
$t_{1/2}$	Half life
C_{max}	Maximum concentration
t_{max}	Maximum time
AUC	Area under the curve
ADMET	Absorption, distribution, metabolism, elimination and toxicity

TPGS	d-α-Tocopheryl polyethylene glycol 1000 succinate
P-gp	P-glycoprotein
GLUT	Glucosyltransferase and glucose transporter
NaTC	Sodium taurocholate
HPCD	Hydroxypropyl-β-cyclodextrin
CMC-Na	Carboxy methylated cellulose sodium
PVP K30	Polyvinylpyrrolidone K30
PEG	Polyethylene glycol
CsA	cyclosporine A
TEER	Transepithelial electrical resistance
KBR	Krebs-Bicarbonate Ringer's solution
RT-PCR	Reverse transcription-polymerase chain reaction

CHAPTER 1: Introduction

1.1 Overview

Our cells are innately infused with differences that cannot be ignored. The phrase "every cell has a sex" captures the essence of how fundamentally different men and women are when it comes to health and disease. However, our sex differences have vastly been ignored when it comes to health research. Female subjects have historically been excluded from toxicology or biomedical research for several reasons, especially given the complexity of their menstrual cycles and risks of teratogenicity. Ignoring female participants from clinical trials have somehow backfired and results to the deaths of at least one in five women, directly related to drug use (Vadam, 2014). The U.S. General Accounting Office (GAO) also reviewed that eight of the ten drugs withdrawn from the market were due to greater risks of adverse effects in women (GAO, 2001).

Progress has finally been made since then when the U.S Food and Drug Administration (FDA) outlined that both sexes should be represented in all phases in clinical trials to avoid undetected sex differences in drug efficacy and side effects (Kando., 1995, Beierle et al., 1999, Soldin et al., 2011). However, there is still a long way to go. In a 2014 study, researchers reported that only 39% of the participants involved in the medical studies were women. More recently, formerly regarded 'inert' excipients reportedly have a sex-related influence on the bioavailability of BCS Class III drugs, a number of which have been proposed to be eligible for biowaivers (waivers from *in vivo* bioequivalence testing) by The World Health Organization (WHO).

Based on the aforementioned data, sex equality has been warranted in clinical as well as pre-clinical research. Better understanding of the reasons behind the sex difference in drug response can provide beneficial information in drug design and formulation strategy. This can also can be considered in the context of personalized medicine.

1.2 Sex Differences in Physiology

John Gray's best seller "*Men Are from Mars, Women Are from Venus"* stated that most common relationship problems between men and women are the result of fundamental psychological differences between the sexes. It is true there are significant physiological differences between men and women, such as body weight, percentage of body fat, body water volume, plasma volume and organ blood flow. These can be implicated in a differing response to medication (Beierle et al., 1999).

1.2.1 Body Composition

The differences between the men and women in body composition are well known (showed in Table 1.1): males typically have proportionately more muscle mass, a higher bone mass and a lower percentage of body fat than women (Brown, 2008).

Body mass is a visible example of a distinction between the sexes. Physical size plays a vital role, but differences go beyond size. Namely, a woman is not a small man. Endocrinological events, such as the onset of puberty in boys and girls, occur at various ages, resulting to variations in body fat, height and growth. Each factor can influence the usage of drugs. For example, the differences in percentage of fat and water between men and women mainly cause the sex-related differences in drug distribution, especially for the highly water-soluble or lipid-soluble medicine. On the other hand, various factors come together to create the anatomic, physiologic, biochemical and endocrine differences between men and women that can influence drug disposition and response.

Table 1.1 Select physiological parameters and their values for men and women.

Parameter	Adult Male	Adult Female
Mass (kg)	73	60
Height (cm)	176	163
Body Surface Area (m2)	1.90	1.66
Fat (kg)	14.6	18.0
Ventilation Rate	22.9	18.5
Cardiac Output (L/min)	6.5	5.9
Tissue Mass (g, varies with age)		
Liver	1,800	1,400
Lung	500	420
Kidneys	310	275
Fat (storage fat)	14,600	18,000
Blood Flow Rate (% Cardiac Output)		
Liver	25.5	27
Kidneys	19	17
Fat	5.0	8.5
Skeletal muscle	17	12

(Extracted from ICRP, 2002. Basic Anatomical and Physiological Data for Use in Radiological Protection Reference Values. ICRP Publication 89. Ann ICRP. 32:3-4), (Brown, 2008).

1.2.2 Cardiac Output

Cardiac output, also known as regional blood flow, is similar for both sexes between 18 and 44 years of age. The distribution of regional blood flow, is similar for men and women for some organs, such as adrenal, bone, brain, lung and skin, but different for others. For example, the percent of cardiac output in liver was 25% in men but 27% in females, reflecting sex-based differences in body composition (ILSI, 1994).

1.2.3 Sex-specific Condition: Sex Hormones

There have been numerous evidences that support the argument that female sex hormones influence drug-metabolizing pathways, increase drug accumulation and/or decrease elimination of some drugs. For instance, estrogen can bind the membrane ion channels and receptors alongside modulate CYP1B1 expression (Nicolson et al., 2010, Tsuchiya et al., 2004). It is further exhibited that female steroid hormones and prolactin can result in a two- to ten-fold increase of inflammatory diseases in women than men (Ostrom, 2006).

1.3 Sex Variations in Drug Pharmacokinetics

All the sex variations mentioned above in physiological parameters could result to sex-related differences in drug response or pharmacokinetics. The basic stages of pharmacokinetics, namely absorption, distribution, metabolism and elimination (ADME), were described in Figure 1.1.

During the period from 1977 to 1995, 26 studies were submitted to U.S. FDA because of the sex-dependent differences in bioequivalence (Chen et al., 2000, Coker, 2008, Nicolas et al., 2009). The number of studies and available sample sizes in these studies were limited, however, the understanding of sex differences in medicinal drug toxicology and pharmacology was expanding (shown in Table 1.2). FDA regulations and guidance were then placed to ensure that both sexes are represented in all phases of clinical trials, and that medical products are labelled to alert physicians and patients to any differences in the way in which men and women respond to a drug.

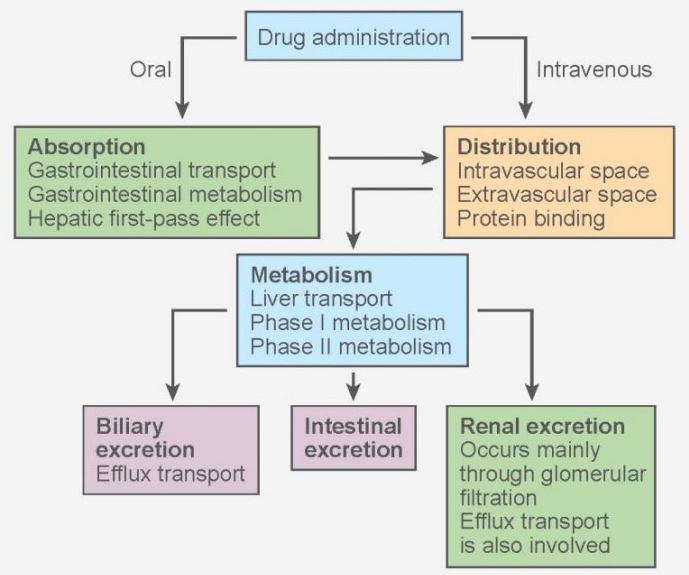


Figure 1.1 The process of absorption, distribution, metabolism and elimination (ADME)**,** (Undevia et al., 2005).

Table 1.2 Sex-specific differences in the pharmacokinetics of drugs.

Drug	Sex Differences	Reasons	Reference
nimodipine	higher AUC_{0-480} and C_{max} in female rats	lower oral clearance in female rats	(Liu et al., 2005)
glucose	lower absorption during the first hour and higher absorption in the last hour of a three-hour OGTT in female	different body sizes and plasma glucose levels, and β-cell fuction from OGTTs should be taken into account	(Anderwald et al., 2011)
	early glucose absorption was lower in females with i-IGT		(Faerch et al., 2013)
metronidazole	higher distribution in males compared with females	water-soluble drugs; lower total body clearance(TBC) and volume of distribution in girls as higher content of water in men	(Carcas et al., 2001)
flouroquinolones	significantly higher AUC_{0-480} in girls(44.8±39.8) than in boys(16.9±5.00)		(Zulfiqar et al., 2008)
pantoprazole	higher AUC in women(8015.4±1056.8) than men(5787.6 ±401.2)		(Miriam et al., 2011)
losartan	larger AUC in females than in males		
rocuronium	longer duration in women compared with men	lipid-soluble drugs; higher content of fat in women	(Xue et al., 1997)
vecuronium			(Houghton et al., 1992)
diazepam	1.67% free fraction found in females while 1.46% shown in men volunteers; larger distribution found in females	plasma binding was similar among men and women, however, concentration of sex hormones impacted the protein systems responsible for drug binding, protein binding contribute to sex-specific differences during the menstrual cycle	(Ochs et al., 1981)
lidocaine	higher distribution was shown in females when compared with males		(Walle et al., 1994) (Soldin and Mattison, 2009)
warfarin			
testosterone			
phenytoin			(Ueno, 2009)

Table 1.2 (*Continued*)

Drug	Sex Differences	Reasons	Reference
metoprolol	lower distribution, higher clearences and shorter $t_{1/2}$ were shown in men than in women	plasma binding was similar among men and women, however, concentration of sex hormones impacted the protein systems responsible for drug binding, protein binding contribute to sex-specific differences during the menstrual cycle	(Luzier et al., 1999)
quinine			(Terrell et al., 2010)
methylprednisolone			(Silvaggio and Mattison, 1994)
propranolol	clearence was smaller in women, and $t_{1/2}$ was decreased in women compared to men but does not appear to be altered during pregnancy		(Nicolson et al., 2010)
zolmitriptan	higher bioavailability in women after both 5mg oral dosing and intravenous dosing	sex differences in first-pass metabolite, as the plasma concentration in women was higher with relatively higher levels of active metabolite 183C91 in men	(Seaber et al., 1998)
diltiazem	higher C_{max} and longer t_{max} in female rats than males	10-fold higher concentration of DAD and 10-fold lower concentration of DMD in females	(Los et al., 1996)
schizandrin	higher C_{max} and AUC_{0-480} were found in female rats compared with male rats	sex differences in the level of metabolism (CYP3A and CYP2C11)	(Xu et al., 2008)
γ-schizandrin			(Zhao, 2010)
letrozole	AUC and $t_{1/2}$ in females were 3-fold and 4-fold higher than in males	letrozole metabolized more extensively in male rats	(Liu et al., 2000)
heparin	longer duration in women compared with men	lower clearance in females	(Campbell et al., 1998)
cefotaxime			(Terrell et al., 2010)
ciprofloxacin			(Waxman and Holloway, 2009)

Table 1.2 (*Continued*)

Drug	**Sex Differences**	**Reasons**	**Reference**
aspirin	higher clearance and shorter $t_{1/2}$ in women	cleared more rapidly in women	(Aarons et al., 1989)
chlordiazepoxide	higher AUC was found in men than in women	sex differences in metabolism	(Greenblatt et al., 1977)
midazolam	lower AUC in women compared with men	lower level of CYP3A expression in males	(Gorski et al., 1998)
atenolol	lower oral clearance and volume of distribution in women	not clear, due to the pharmacokinetcis but not pharmacodynamic differences	(Custodio et al., 2008)
flurazepam	higher AUC of its major blood metabolites namely N-1-hydroxyethylfurazepam and N-1-desalkylflurazepam in women	sex differences in metabolism transporters or proteins	(Cooper et al., 1984)
ranolazine	plasma concentrations in female rats were significantly higher than those in male rats. Drug exposures based on C_{max} and AUC in female rats were roughly 2-to 3-fold of those in male rats. $t_{1/2}$ and MRT in male rats were shorter than those in female rats. The recoveries in urine and bile of female rats were also markedly higher than those in male rats. Tissue concentrations in female rats were also markedly higher than those in male rats.	marked sex difference in ranolazine pharmacokinetics in rats, the main reason may be due to sex-related differences in metabolism.	(Liu et al., 2003)
levofloxacin	higher C_{max} was found in females while larger AUC_{0-480} was shown in males	differences in body sizes by sexes	(Almeida et al., 2005)

Table 1.2 (*Continued*)

Drug	Sex Differences	Reasons	Reference
daidzein	higher AUC_{0-480} was attained in women, but no differences between premenopausal and postmenopausal women in $t_{1/2}$, t_{max} and AUC	———	(Cassidy et al., 2006)
genstein	2-fold higher oral bioavailability of total genstein in females with 4-fold higher plasma geinstein glucuronide concentrations and higher biliary exretion	not because of the sex differences in hormonal changes	(Kulkarni, 2010)
fentanyl	lower urinary excretion in women than in men	higher glomerular filtration rat(GFR) in males compared to female groups	(Berg, 2006)
taurocholate	renal clearance was markedly decreased in male rats		(Schlattjan et al., 2005)
torasemide	lower clearance in females with higher AUC and C_{max}		(Werner et al., 2010)
celastrol	higher absolute bioavailability in female rats after oral administration	mechanism for absorption and metabolism	(Zhang et al., 2012)
ofloxacin	higher AUC and C_{max}, and lower total body clearance(TBC) and volume of distribution in girls	———	(Hassan, 2008)
fluconazole	higher plasma levels in women than in men	sex differences in the level of CYP3A4, CYP2C9, CYP2C19	(Carrasco-Portugal Mdel and Flores-Murrieta, 2007)
clindamycin			(Lujan and Dicarlo, 2008)
acebutolol	larger AUC was found in females	———	(In et al., 1990)
ethanol	smaller distribution was found in women while lower AUC was shown in men	as the ethanol is ingested and men metabolize more in first pass metabolism	(Gaudry et al., 1993)

1.3.1 Absorption

The first step for a drug to reach its site of action is its absorption (for drugs not administered intravenously). The factors influencing absorption are drug- and route-specific but also sex-dependent.

The cases that illustrate sex differences in drug absorption include aspirin and naratriptan. To be specific, the oral administration of aspirin to healthy adult male and female young volunteers resulted in quicker absorption in females than in males (mean absorption times of 16.4 and 21.3 min, respectively) (Aarons et al., 1989, Ho et al., 1985). Sex differences were also found in the oral bioavailability of naratriptan, with higher peak concentrations in females than in males (Mattison and Zajicek, 2006). There are several possible reasons behind these sex-related differences in drug absorption, one being the role of physiological parameters (shown in Table 1.3).

Moreover, iron and alcohol are two important chemicals that exhibit significant differences in gastrointestinal absorption between males and females. Gastric alcohol dehydrogenase activity is higher in males than in females. Decreased gastric alcohol dehydrogenase has been shown to result in a higher percentage of absorbed alcohol concentrations (Parlesak et al., 2002). On the other hand, in preadolescent males and females, it has been shown that 45% of ingested iron is incorporated into erythrocytes by females compared with 35.2% in males (Arthur et al., 1984, Jacobs, 1976).

In general, oral drug administration is the route of choice in the daily practice of pharmacotherapy, especially in outpatient settings. It is therefore important to consider that the ingestion of food, food interactions (e.g. grapefruit juice) (Lilja et al., 2005), gut motility and transit time, gut pH, biliary secretion and gut flora (enterohepatic circulation and the impact of oral contraceptives) can have major influences on drug absorption (Soldin and Mattison, 2009).

Table 1.3 Sex differences in physiological parameters which influence absorption**,** (Soldin and Mattison, 2009).

Parameter	**Physiologic Difference**	**Pharmacokinetic Impact**
Gastric pH	Acidity M>F>pregnant F	Altered absorption of acid/bases depending on specific drug ionization. In pregnancy decreased absorption of weak acid.
Gastric Fluid Flow	M>F	Higher absorption in males
Intestinal Motility	M>F>pregnant F	Absorption increased in males
Gastric Emptying	M>F>pregnant F	Absorption, gastric hydrolysis increased
Dermal Hydration	Increased in pregnant F	Altered absorption in pregnant F
Dermal Thickness	M>F	Absorption decreased in males
Body Surface Area	M>pregnant F>F	Absorption increased when surface area larger
Skin Blood Flow	Increased in pregnant F	Absorption increased
Pulmonary Function*	M>pregnant F>F	Pulmonary exposure increased in males
Cardiac Output*	M>pregnant F>F	Absorption increased in males

M = male; F = female
*normalized for body surface area

1.3.2 Distribution

Once absorbed, most drugs bind to plasma proteins that are specific for some aspect or structural feature of the drug. The distribution of a drug is affected by multiple body composition parameters (shown in Table 1.4). Sex differences in these parameters may account for differences in the concentration of a drug at the target site and result in varying responses.

Table 1.4 Sex differences in physiological parameters which influence distribution**,** (Soldin and Mattison, 2009).

Parameter	**Physiologic Difference**	**Pharmacokinetic Impact**
Plasma Volume	Pregnant F>M>F	Decreased concentration in pregnancy
Body Mass Index (BMI)	M>F	Higher in men
Average Organ Blood Flow	Pregnant F>M>F	Higher in pregnant women
Total Body Water	M>Pregnant F>F	Decreased concentration
Plasma Proteins	M,F>pregnant F	Free concentration increases in pregnancy
Body Fat	pregnant F>F>M	Increased body burden of lipid-soluble drug in women
Cardiac Output	M>pregnant F>F	Increased rate of distribution in men

On average, men have higher total body water, extracellular water, intracellular water, total blood volume, plasma volume and red blood cell volume than women. The larger proportions of body fat in women, especially in pregnant women, may increase the body burden of lipid-soluble, slowly metabolized toxicants. Differences in body fat and organ blood flow in women have been implicated in the faster onset of action and prolonged duration of neuromuscular blockade in women (e.g. vecuronium and rocuronium bromide) (Houghton et al., 1992, Xue et al., 1997). Differences in body fat content and in protein binding are responsible for sex-

related pharmacokinetic differences in the distribution of diazepam, where females have been shown to have larger volumes of distribution than males due to higher free fraction (Ochs et al., 1981).

Using a previous human study as an example, women exhibited a significantly lower peripheral volume of distribution (V_p/F) (3.33±0.38 L/kg for women; 5.49 ±0.31 L/kg for men) after the oral administration of *(S)*-metoprolol and *(R)*-metoprolol, respectively (Luzier et al., 1999). Furthermore, women were shown to have significantly higher C_{max} values compared with men. A greater drug exposure was observed in women as evident by greater AUC in women than in men as well (shown in Figure 1.2).

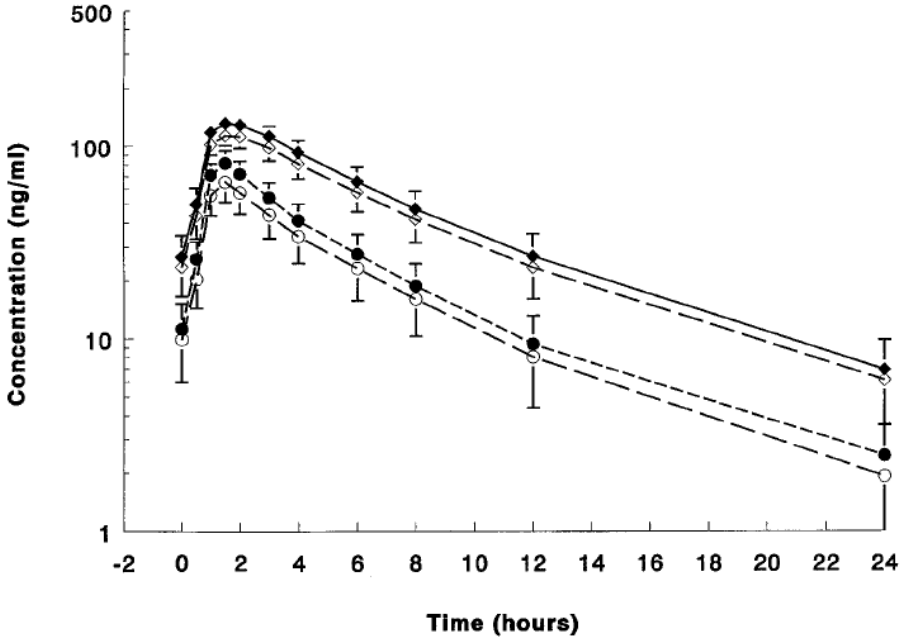


Figure 1.2 Fitted plasma concentration versus time profile for (R)-metoprolol (open symbols) and (S)-metoprolol (solid symbols) in male (circles) and female (diamonds) subjects (Mean±S.D, n=10), (Luzier et al., 1999).

1.3.3 Metabolism

Drug metabolism (biotransformation) occurs predominantly in the liver, as well as in extra hepatic sites such as the intestinal tract, lung, kidney and skin. Despite large variations in drug metabolism in individuals, correction for height, bodyweight, body surface area and body composition eliminates most 'sex-dependent' differences. Sex-dependent differences in drug metabolism, however, have been observed mainly due to transporters and enzymes expressed on the hepatocytes. It was reported that drugs metabolized by phase I, phase II (conjugative) and by combined oxidative and conjugation processes are usually cleared faster in men compared to women (Schwartz, 2007, Schwartz, 2003).

Sex-specific difference in hepatic metabolism was first observed in the case of antipyrine in 1971 (Omalley et al., 1971). After that, some other studies have also reported sex-based differences in the bioavailability of drugs due to drug metabolism, such as heparin (Campbell et al., 1998), chlordiazepoxide (Greenblatt et al., 1977), midazolam (Gorski et al., 1998), flurazepam (Cooper et al., 1984) and nimodipine (Liu et al., 2003).

Interestingly, dose-related sex differences were found in some drug metabolisms. Using zolmitriptan as an example (summarized in Figure 1.3), the bioavailability of zolmitriptan was significantly higher in women than men after both 5mg oral dosing and intravenous dosing. However there were no reported sex differences in oral bioavailability with a dose of 2.5mg (Seaber et al., 1998). This sex-related variation was smaller than the finding in the previous report which also demonstrated sex difference in the bioavailability of zolmitriptan after 10mg oral administration (Seaber et al., 1997). It therefore stands a dose-related manner in the bioavailability of zolmitriptan. The reason for this sex-dependent difference was assumed to be most likely explained by a difference in first-pass metabolism (Seaber et al., 1996), as the plasma concentrations of zolmitriptan in women were higher than in men with relatively higher levels of the active metabolite 183C91 in men.

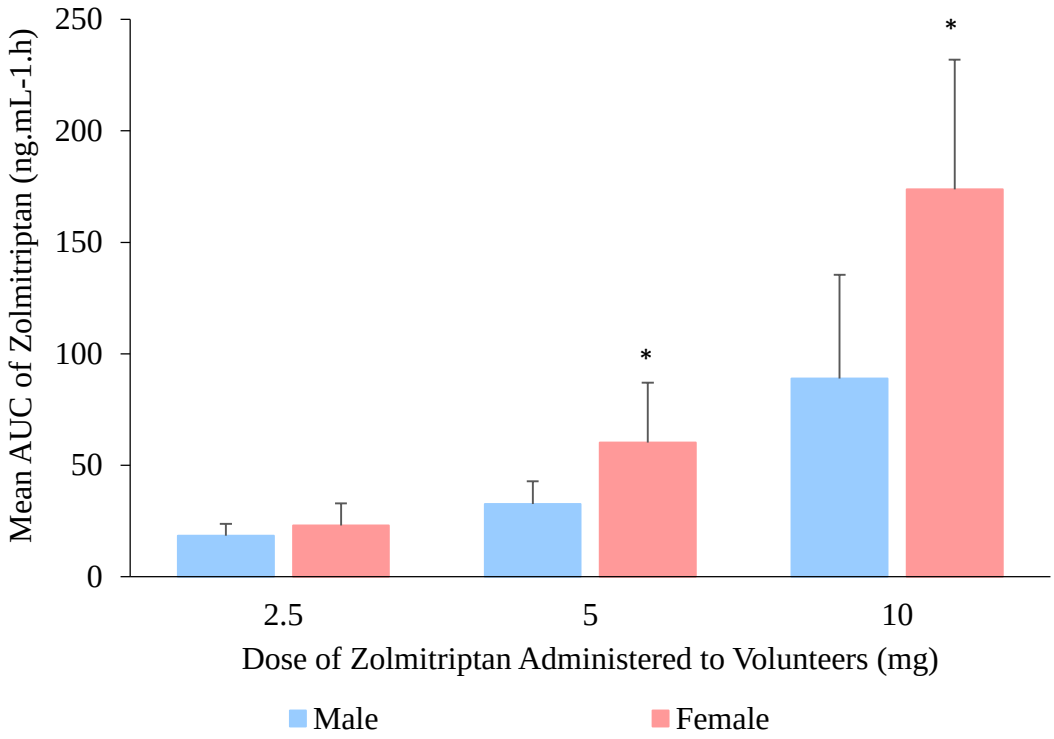


Figure 1.3 Mean $AUC_{0-\infty}$ after 2.5mg, 5mg and 10mg zolmitriptan in men and women (Mean±S.D., n=10), (Seaber et al., 1998, Seaber et al., 1997). * Values are statistically different between the male and female groups at $p<0.05$.

1.3.4 Elimination

The kidney is the major organ of drug excretion of either the parent drug compound or the drug metabolites. There are known sex differences in all three major renal functions (namely glomerular filtration, tubular secretion and tubular reabsorption) resulting in generally higher renal clearance in men than in women (Gaudry et al., 1993, Berg, 2006).

For example, the clearance of acetaminophen was noted being slower in females than in males (Slattery et al., 1987). It is possibly due to the increases in renal blood flow and glomerular filtration increase the elimination rate of drugs cleared by the kidneys.

Renal blood flow, glomerular filtration, tubular secretion and tubular reabsorption are all greater in men than in non-pregnant women, however, changes in renal blood flow, the glomerular filtration rate, hepatic blood flow, bile flow and pulmonary function may alter maternal elimination of a drug in women during gestation.

1.4 Sex-specific Influence of Excipients on Drug Bioavailability

This issue regarding sex inequality in drug response becomes more complicated when excipients are co-formulated in the medicine.

Celastrol, a chemical compound isolated from the root extracts of *tripterygium wilfordii* (Thunder god vine) and *celastrus regelii*, exhibits anti-inflammatory (Kim et al., 2009) and anti-cancer (Lee et al., 2001, Byun et al., 2009) ability. The bioavailability of celastrol was the same in male and female rats. However, the presence of carboxymethylcellulose sodium (CMC-Na) three-fold increased the celastrol bioavailability in female rats, while there was no change in male rats (summarized in Figure 1.4) (Zhang et al., 2012).

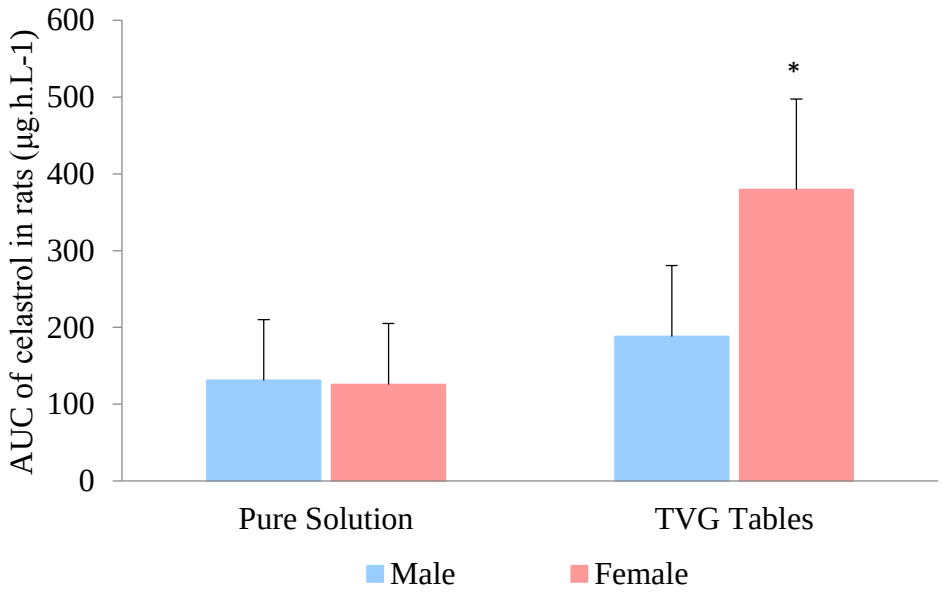


Figure 1.4 The bioavailability of celastrol in male and female rats after administration with two celastrol formulations (Mean±S.D). * Values are statistically different between pure solution and TVG table groups.

In another study, the area under the curve (AUC) of the drug γ-schizandrin following oral administration of pure γ-schizandrin solution (dissolved in water) was twenty-times higher in male rats compared to females. Unexpectedly, an opposite trend was observed when γ-schizandrin was administered as a solid dispersion with polyvinylpyrrolidone K30 (PVP K30) or in a capsule prepared in-house, where AUC in female rats was six-fold higher than in male ones from both γ-schizandrin formulations (shown in Figure 1.5). Although the mechanism of this sex-based difference has not been identified, the surprising influence on γ-schizandrin bioavailability could have been caused by the excipients in the formulations, such as PVP K30 in the solid dispersion or starch in the capsule formulation (Xu et al., 2008, Zhao, 2010).

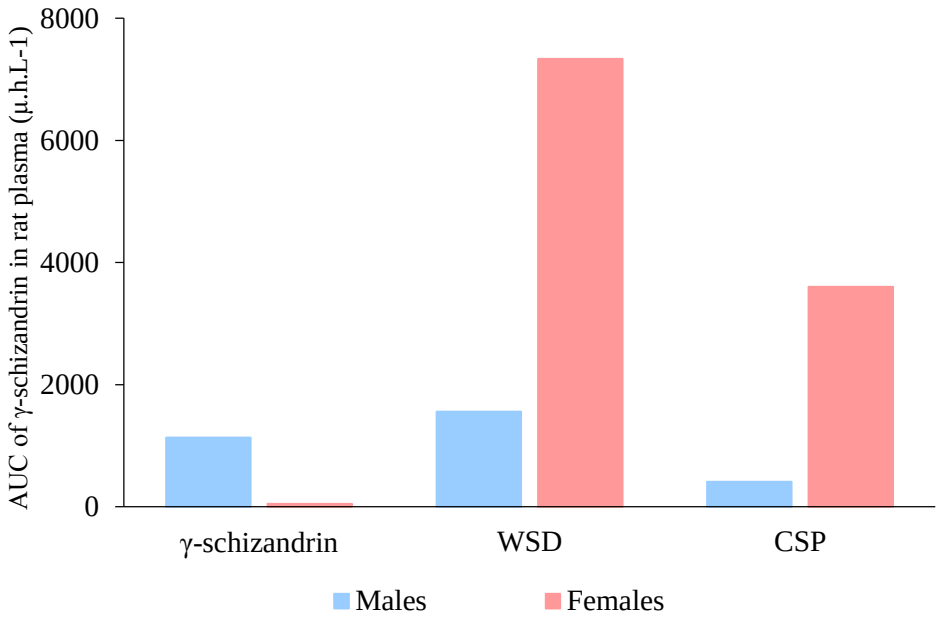


Figure 1.5 $AUC_{0-\infty}$ of γ-schizandrin after administered pure γ-schizandrin solution and twoγ-schizandrin formulations in male and female rats.

In our laboratories, we have investigated the influences of polyethylene glycol 400 (PEG 400) on oral ranitidine bioavailability in both men and women. Men and women had similar ranitidine bioavailability when they took the pure ranitidine water solution, however, orally addition of PEG 400 increased the bioavailability of ranitidine in men but not in women (human data was described in Figure 1.6) (Ashiru et al., 2008). This unexpected sex-dependent influence of PEG 400 on ranitidine was also revealed in the animal model using Wistar rats (Afonso-Pereira et al., 2016). Moreover, intravenous PEG 400 administration could not induce the bioavailability of ranitidine in both male and female rats. This may provide the reason behind the sex-related impact of PEG on ranitidine in the gastrointestinal (GI) tract (unpublished data).

PEG 400 is a widely used excipient which is typically employed as a solubility enhancer to improve the dissolution and subsequent bioavailability of poorly-soluble drugs. Other aforementioned excipients such as CMC-Na, PVP K30 and starch are also commonly co-formulated in the products widely used in the market. These excipients are added to formulations to aid in further processing of the materials into its final dosage form and to achieve an optimum absorption and therapeutic effect (Badawy et al., 2006). Most “inert” materials commonly used in the design of oral dosage forms are assumed to not influence drug absorption. It has, however, recently been reported that these inert excipients are able to affect drug bioavailability via the alteration on the physiological characteristics and membrane transporters in GI tract (more information shown in section 1.4.1 and 1.4.2). This may possibly have a sex-dependent manner (more information shown in section 1.5), and thus, further contribute to the fact that excipients have a sex-specific effect on the bioavailability of the drug.

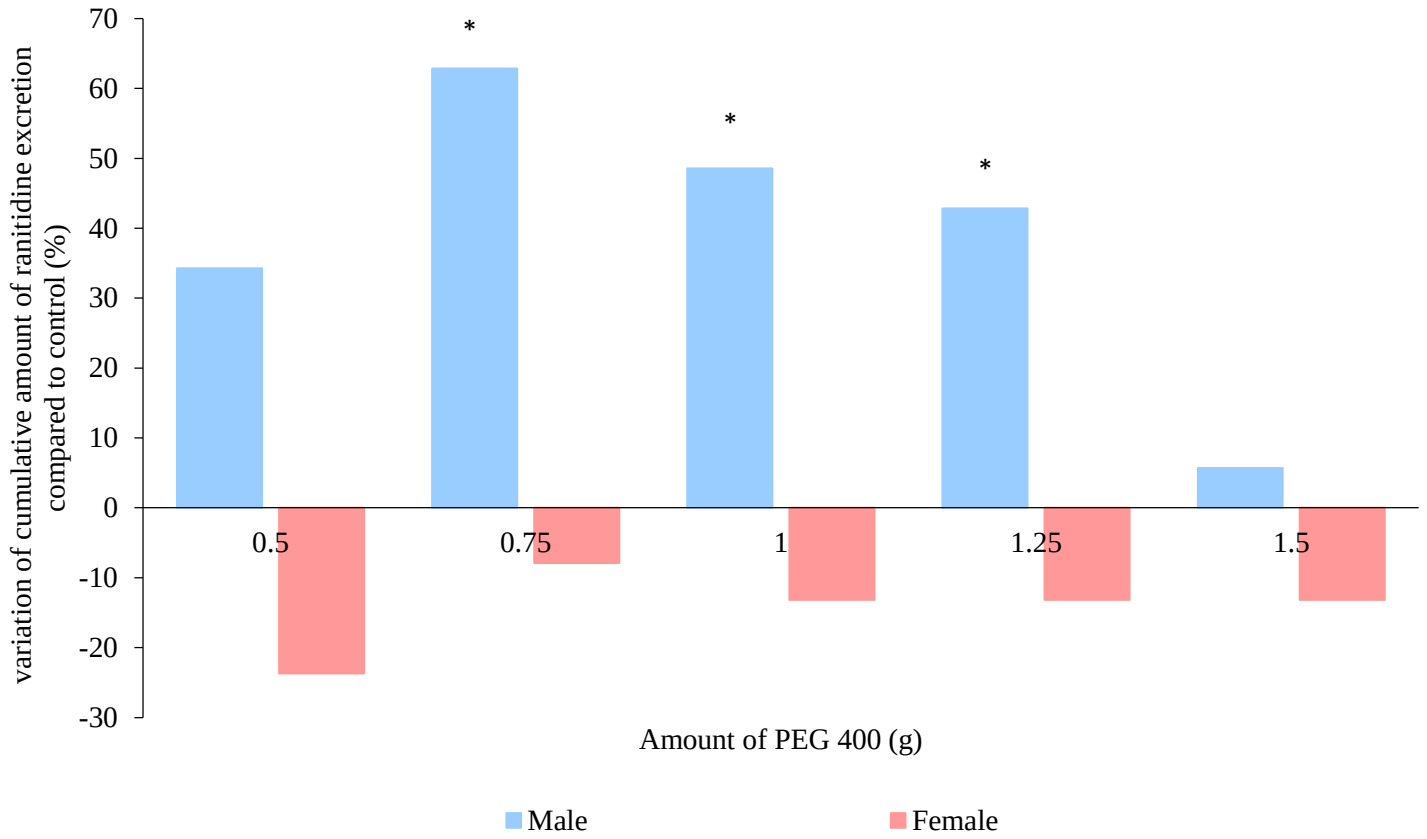


Figure 1.6 Change in ranitidine bioavailability in the presence of different doses of polyethylene glycol 400 in male and female volunteers, n=6 (Ashiru et al., 2008). * Values are statistically different between the control and PEG 400 groups at $p<0.05$.

1.4.1 Physiological Characteristics

Pharmaceutical excipients can influence the absorption, distribution and clearance of the drugs due to their effects on a variety of physiological characteristics of GI tract or blood fluid rate. For example, the emulsifying agent, cholesterol, increases the fluidity of cancer cell membranes (Baggetto and Testa-Parussini, 1990). The suspending agent, carrageenan, induces inflammatory reactions in rats and mice (Halici et al., 2007, Farges et al., 2006), whilst the filler, mannitol, reduces small intestine transit time in a dose-dependent manner (Adkin et al., 1995b, Adkin et al., 1995a).

1.4.2 Membrane Transporters

Transporter proteins are integral proteins that function via either facilitated diffusion, or active, energy-dependent mechanisms to mediate transcellular flux of xenobiotics and nutrients (Amidon et al., 1995). These transporters were reportedly modified by some active pharmaceutical excipients.

For instance, surfactants have been found to reduce membrane viscosity and increase elasticity (Martin et al., 1978). This results to an enhanced absorption of compounds by both the paracellular and the transcellular pathways (Tomita et al., 1988). In addition, changes in membrane fluidity can alter the microenvironment of the apically oriented transmembrane domains, and subsequently, alter substrate recognition, substrate binding and/or ATPase activity of efflux of transporters (P-gp) or the function of influx transporters (PEPT-1) (Ferte, 2000).

1.5 Sex Differences in Gastrointestinal Tract

The gastrointestinal tract is comprised of the stomach and intestines, and is divided into the upper and lower gastrointestinal tracts. Sex variations were reported in some human and animal studies in terms of its physiological characteristics and membrane transporters.

5.1.1 Gastrointestinal Anatomy

The mucosal surface area of the stomach from 222 volunteers revealed that women (783 cm^2) have smaller stomachs than men (850 cm^2) in a post-mortem study (Cox, 1952). The anatomical features have been reviewed in Figure 1.7.

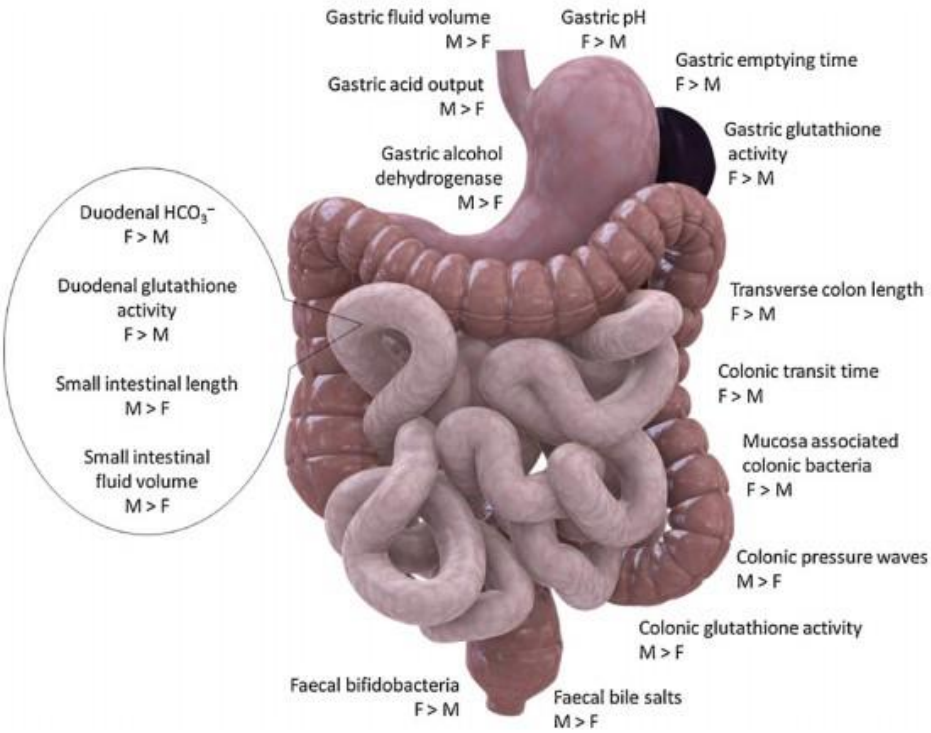


Figure 1.7 Key sex differences at the level of gastrointestinal tract affecting oral drug delivery and bioavailability (M=Male, F=Female), (Freire et al., 2011).

1.5.2 pH of Gastrointestinal Lumen

The fasting gastric pH was reportedly higher in women when compared with men, and researchers purposed it was due to the two-fold increase of basal acid output in men without taking meals (Feldman and Barnett, 1991). The secretion of acid was also substantially higher in males in the fed state (Prewett et al., 1991). Interestingly, there were no sex differences shown in the pH of duodenum and jejunum, although these studies were conducted among a small number of subjects (Lindahl et al., 1997, Perez de la Cruz Moreno et al., 2006). The prevalence of duodenal ulcers was further identified to be lower in women than in men (Johnson et al., 1992).

There were few studies on the sex-related differences in the pH of colon, and the majority of the studies did not exhibit any statistically significant differences (McDougall et al., 1993). However, faecal pH is higher in women than in men owing to a higher incidence of methane producing bacteria in women (49%) than men (33%) (Stephen et al., 1986, Pitt et al., 1980).

1.5.3 Volume of Fluid

Sex-associated variations could not only be found in the pH values of gastrointestinal lumen, but also the volumes of lumen fluid. In a study involving 8 men and 5 women, post-mortem fluid volumes were measured in the stomach, small intestine and proximal colon. It stands that the fluid volume in stomach and intestine were higher in males than in females, whereas there no differences in the volume of fluid in colon were identified between the sexes (Gotch et al., 1957). Since then, it has been noted that postprandial changes in gastric volumes were higher in males than females (Bouras et al., 2002).

1.5.4 Composition of Fluid

Considerable attention has been paid to the characterization of the luminal fluids in last decade (Kalantzi et al., 2006, Clarysse et al., 2009). However, there were previously limited knowledge regarding the composition of the gastric and intestinal fluids by sex, except in saliva. The composition of saliva has been shown to vary between men and women (Bales et al., 1990, Laine et al., 1991). Female sex hormones may be responsible for such differences as salivary composition has been shown to fluctuate during pregnancy (Laine et al., 1988). This phenomenon was also identified in women who take oral contraceptives (Laine et al., 1991).

In addition, the differences in bile acid composition and secretion between male and females is well documented. The ratio of primary to secondary bile acids in the gallbladder is lower in young adult women when compared with men (Fisher and Yousef, 1973). Moreover, the composition of bile fluid also appears to change during the menstrual cycle and pregnancy in women (McMichael and Potter, 1980).

1.5.5 Gastrointestinal Motility

A number of studies have highlighted differences in gastrointestinal motility (usually represented with transit time) by sexes, and these differences have been well reviewed in previous reports (Freire et al., 2011). Higher total gastrointestinal transit time has been shown in females (91.7±12.8h) than in males (44.8±4.3h), with clear differences seen mainly in gastric emptying time and colonic remain time (Stephen et al., 1986). When it comes to the variability in the small intestine transit times between sexes, the results are controversial. No differences have been reported in some studies (Isner et al., 1992), whist other reports showed longer small intestine transit time in females (Graff et al., 2001, Sadik et al., 2003). In contrast, it has been concluded that there is a markedly slower colon transit in women compared to the men (Jung et al., 2003). More data on values for transit in the right, left and sigmoidal colon have been exposed; longer transit times in right and left colon was found in female volunteers compared to male groups (Metcalf et al., 1987). A lower pressure activity in women was also demonstrated when compared with their male counterparts. In addition, there were variations in the transverse and descending colon between the sexes (Rao et al., 2011).

1.5.6 Membrane Transporters

Apart from physiological characteristics, membrane transporters have also been shown to play an essential role in drug absorption (Zhang et al., 1998). A number of these membrane transporters have been reported of their sex-associated manners, leading to sex-related differences in drug bioavailability.

Extraction of chemicals from the intestinal lumen is achieved by passive diffusion, also accomplished by a variety of transporters, including OCT-1, OCT-3, OCTN-1, OCTN-2, PEPT-1, PEPT-2 and OATPs. Likewise, there are apical efflux transporters on enterocytes (BCRP, P-gp and MRPs) that prevent the entry of chemicals into the systemic circulation and are often responsible for the poor oral bioavailability of pharmaceuticals (shown in Figure 1.8).

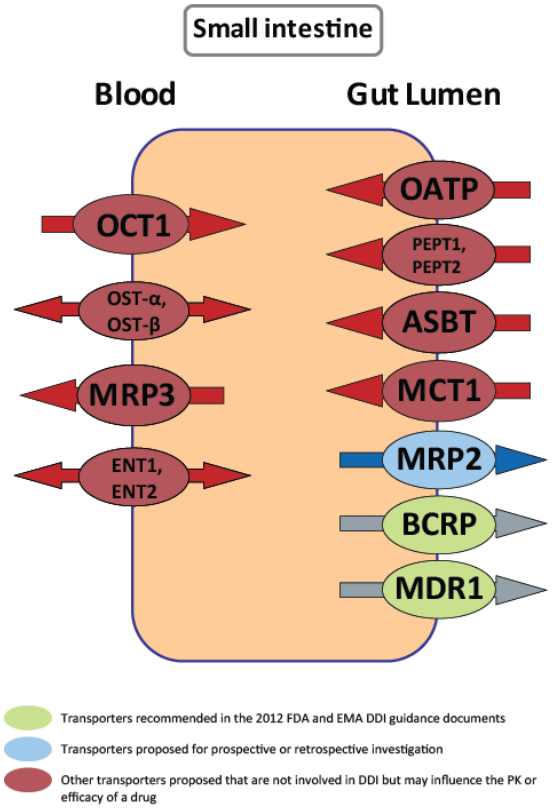


Figure 1.8 Diagram of major drug transporters on the intestinal epithelia, (derived from XenoTech).

There were a few studies that reported sex-related differences in the expression of uptake transporters in the intestine (shown in Table 1.5). One of the best studied influx transporters in the intestine was OATPs. Of the OATPs, only OATP2B1 has relatively high expression in intestine. It is generally believed that most xenobiotics are mainly absorbed from the intestine by simple diffusion. However, OATP2B1 is still a vital uptake transporter in the intestine and has demonstrated a higher expression in the jejunum in males compared with female rats (Cheng et al., 2005).

On the other hand, compared to the influx transporters, the influence of sex on the efflux transporters in the intestine has been better investigated via both indirect and direct methods. Ballent *et al* (2011) studied the transport of P-gp substrate ivermectin in the presence or absence of P-gp inhibitor PSC833 across the intestinal sacs of male and female Wistar rats. The results demonstrated ivermectin accumulation in the everted gut sacs was higher in female compared with male intestine, hereby suggesting higher P-gp expression in the intestine of male rats than females (Mariana et al., 2011). This finding was in line with some previous reports indicated men may have a significantly higher level of P-gp in the jejunum compared with women (Schuetz et al., 1995a).

For another two efflux transporters, namely MRPs and BCRP, similar protein expression was found between males and females in the small intestine. However, the pharmacokinetics of nitrofurantoin, a specific BCRP substrate, was examined after oral or intravenous administration in rats and mice treated with chrysin (a typical BCRP inhibitor), following BCRP, mRNA levels in the intestine were measured. The results validated lower expression of BCRP in female rats and mice (Atsushi et al., 2009), which supported a study conducted by Yuji *et al.* that outlined the different sex-related BCRP mRNA expressions in rat and mouse intestinal tissues (Yuji et al., 2005)

Table 1.5 Sex-based differences in typical transporters in the gastrointestinal tract.

Location	Transporters	Sex Differences	Content	Reference
Intestine	PEPTs	M=F	the thyroid hormone regulation of PEPT1 and PEPT2 mRNA in rodents has been studied, and showed PEPTs have no significant difference between sexes (rodents)	(Lu and Klaassen, 2006)
	OATP2B1	M>F	higher DNA expression in jejunum in males (mice)	(Cheng et al., 2005)
	P-gp	unclear	higher absorption of Rhodamine 123 was measured in the intestine of females and higher P-gp expression was shown in females; but higher enterocyte P-gp content in men has been reported; while no differences between the sexes in P-gp expression in the upper duodenum (rats)	(Schuetz et al., 1995a) (Potter et al., 2004) (Paine et al., 2005a) (Mariana et al., 2011)
	BCRP	M>F	lower mRNA expression of BCRP in females (rats and mice)	(Atsushi et al., 2009)
	MRP	M=F	no significant differences in MRP2 between males and females in the quantitative real-timepolymerase chain reaction and Western blot studies (rats)	(Caroline et al., 2008)

1.6 Rationale for the Study

Based on the interest in the previous finding regarding sex-specific influence of PEG 400 on ranitidine bioavailability, the aim of this research project was to clarify the reason behind the unexpected sex-associated phenomenon and to identify more excipients that would express a sex-based effect on the overall drug bioavailability.

In view of the aforementioned findings on the sex differences in the physiological characteristics in GI tract and membrane transporters, alongside the interaction between these factors and "inert" pharmaceutical excipients, we hypothesize the reason behind this sex-associated phenomenon could be related to the mechanisms of ranitidine absorption.

Ranitidine is predominantly absorbed via the paracellular pathway and membrane transporters of which both influx and efflux transporters are involved, (Bourdet and Thakker, 2006). Therefore, the bioavailability-enhancing effect of PEG 400 in males could be due to the opening of tight junctions by PEG 400 and/or its interactions with membrane transporters. It is known that ranitidine is a substrate for the organic cation uptake transporters (OCTs) (Ming et al., 2009, Han et al., 2013), as well as the efflux transporter P-glycoprotein (P-gp) (Cook and Hirst, 1994, Collett et al., 1999). Meanwhile, PEGs are known to inhibit P-gp in a concentration-dependent manner from 0.1 to 20% (w/v) (Hugger et al., 2002, Shen et al., 2006b). Additionally, the activity and expression of P-gp has been reported to be different in males and females (Mariana et al., 2011). Consequently, we contemplated that the observed sex-related influence of PEG 400 on the bioavailability of ranitidine could be due to its interaction with the efflux membrane transporter P-gp. Therefore, a step-by-step investigation was conducted in this study.

The current thesis summarized the following parts of the research project:

- Determination of the influence of PEG 400 on the bioavailability of another two P-gp substrates (fexofenadine and ampicillin) and a non-P-gp substrate (metformin) in male and female rats (Chapter 2: Phase I).

- Assessment of the effect of PEG 400 on drug bioavailability in the presence of a P-gp inhibitor (the immunosuppressive agent cyclosporine A) (Chapter 2: Phase II).

- Extensive investigation into the influence of PEG 400 on the permeation of ranitidine and P-gp activity in four intestinal divisions of male and female rats, with an *in vitro* method (Chapter 3).

- Evaluation of the sex differences on the P-gp protein content and mRNA expression, alongside the influence of PEG 400 on the intestinal P-gp expression in male and female rats (Chapter 4).

- Mechanism investigation into the influence of other commonly-used excipients on the absorption of ranitidine in both male and female rats (Chapter 5: Phase I).

- Exploration the sex-related effect of the aforementioned excipients in Phase I (commonly-used doses in the commercial products) on the *in vivo* bioavailability of ranitidine (Chapter 5: Phase II).

CHAPTER 2: Innate Influences: Do efflux transporters contribute to sex-specific differences of PEG 400 on drug bioavailability?

2.1 INTROUDCTION

The formerly regarded "inert" pharmaceutical excipient, polyethylene glycol 400 (PEG 400) unexpectedly alters the bioavailability of the BCS class III drug, ranitidine, in a sex-dependent manner. We hypothesized that the sex-related influence could be due to interactions between PEG 400 and the efflux transporter P-glycoprotein (P-gp). The investigation was completed in two phases to test this hypothesis.

Phase I: The determination on the influence of PEG 400 on the bioavailability of another two P-gp substrates (fexofenadine and ampicillin) and a non-P-gp substrate (metformin) in male and female rats.

Ampicillin and fexofenadine have been reported to be mediated by the same efflux transporter, P-gp, but differential influx transporter peptide transporters (PEPTs) and organic anion-transporting polypeptide (OATP) transporter respectively (Glaeser et al., 2007, MacLean et al., 2010). Metformin was chosen as it is a non-P-gp substrate and is also transported via the same uptake transporter and organic cation transporters (OCTs) as ranitidine (Chen et al., 2010). Furthermore, all drugs tested in this study are transported via the paracellular pathway. Thus, any possible influence of PEG 400 on the paracellular pathway would be observed, if applicable. (Lafforgue et al., 2008, Alvi and Chatterjee, 2014) (Details of absorption mechanisms and the model drugs are shown in Table 2.1 and Table 2.2).

OATPs, OCTs and PEPTs are the three main families of uptake transporters in the GI tract. In details, OATPs are responsible for the uptake of a wide range of substrates contain steroidal or peptide structural backbones and/or anionic or cationic chemicals (Abe et al., 1999, Hsiang et al., 1999, Cui et al., 2001). OCT transporters mediate the uptake of organic cations that are positively charged at physiological pH, with relatively low molecular weights and also with widely diverse molecular structures (Koepsell and Endou, 2004). PEPTs transports di- and tripeptides into cells, which were first identified as key peptide carriers in the small intestine and kidneys, respectively (Fei et al., 1994, Liu et al., 1995).

If the sex-related influence of PEG 400 occurred only for drugs whose absorption is controlled by the efflux transporter P-gp (fexofenadine and ampicillin in this study), we will then proceed to Phase II. If not, the results will be analyzed and the transporter responsible for those drugs that showed sex differences in the presence of PEG 400 will be regarded as the determining factor behind this sex-related phenomenon. Then, this transporter will be blocked in the following study.

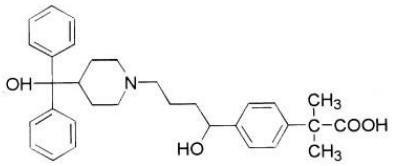
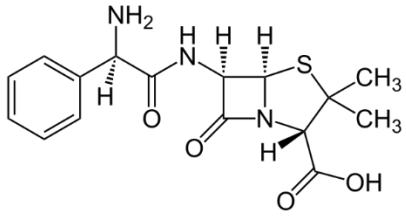
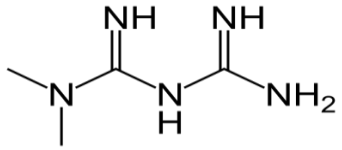
Table 2.1 The substrates and inhibitors for various transporters on the intestinal epithelial membrane.

BCS Class III Drugs	**Influx Transporter**			**Efflux Transporter**		
	PEPT	**OATP**	**OCT**	**P-gp**	**BCRP**	**MRP2**
Ranitidine	×[1][2][3]		√[4]	√[5][6]	×[6]	×[6]
Metformin	×[1][2][3]		√[7]	×[8]	×[1]	
Ampicillin	√[9]		×[1][10][11]	√[12]		
Fexofenadine	×[1][2][3]	√[13]		√[14][15]	×[15]	×[15]

√ stands for the substrates. × stands for this drug is neither inhibitor nor substrates for the transporter.

[1](Konig et al., 2013) [2](Leibach and Ganapathy, 1996) [3](Liang et al., 1995) [4](Muller et al., 2005) [5](Bourdet et al., 2006) [6](Collett et al., 1999) [7](Chen et al., 2010) [8](Song et al., 2006) [9](Sala-Rabanal et al., 2008) [10](Muller et al., 2012) [11](Tsuji et al., 1981) [12](Siarheyeva et al., 2006) [13](Bailey et al., 2000) [14](Chen, 2007) [15](Tahara et al., 2006)

Table 2.2 Physicochemical properties of fexofenadine, ampicillin and metformin.

Model Drugs	Molecular Weight	pKa	Solubility in Water	Molecular Formula	Structure
Fexofenadine	538.13	pKa_1=4.25 pKa_2=9.53	2.44mg/mL	$C_{32}H_{39}NO_4$	
Ampicillin	349.41	pKa_1=2.5 pKa_2=7.3	50mg/mL	$C_{16}H_{19}N_3O_4Na$	
Metformin	165.62	pKa_1=2.8 pKa_2=11.5	>300mg/mL	$C_4H_{11}N_5$	

Phase II: The investigation of the influence of PEG 400 on drug bioavailability in the P-gp 'knock-out' model.

So far, the P-gp blocking animal models were conducted in two methods: 1) using a genetically modified mouse or rats (whose existing specific gene was replaced or disrupted with an artificial piece of DNA); 2) blocking the target protein or mRNA with its inhibitors. In our study, the rats modified with method 2 (blocking P-gp with inhibitors) were used, as the P-gp gene "knock-out" rodent forms were too expensive.

P-gp inhibitors have been intensively studied as potential multidrug resistance (MDR) reversers (shown in Table 2.3 and Table 2.4). Initially, drugs to reverse MDR were not specifically developed for inhibiting P-gp; in reality, they had other pharmacological properties, as well as a relatively low affinity for MDR transporters. An example of this first-generation P-gp inhibitors is verapamil. The second generation included more specific inhibitors that presented lower side-effects such as dexverapamil or dexniguldipine. A third generation of P-gp inhibitors comprises of compounds such as tariquidar with high affinity to P-gp at nanomolar concentrations (Palmeira et al., 2012).

Table 2.3 Classification of P-gp inhibitors.

Generations	**Samples**	**Specificity**	**Limitations**
First Generation	verapamil, cyclosporin A, reserpine, quinidine, yohimbine, tamoxifen, toremifena	Non-selective and low binding affinities.	They are substrates to other transporters and enzyme systems. They are pharmacologically active. They themselves are transported by P-gp.
Second Generation	dexverapamil, dexniguldipine, valspodar (PSC 833), dofequidar fumarate (MS-209)	Higher specificity then first generation inhibitors but interact with other systems.	They are substrates to CYP 3A4 enzyme and other ABC transporters.
Third Generation	cyclopropyldibenzosuberane zosuquidar (LY335979), laniquidar (R101933), mitotane (NSC-38721), biricodar (VX-710), elacridar (GF120918/GG918), ONT-093, tariquidar (XR9576), HM30181	Highest specificity that specifically and potently inhibit P-gp function.	No limitations like the first and the second generation inhibitors.

Table 2.4 Characterization of Typical P-gp Inhibitors.

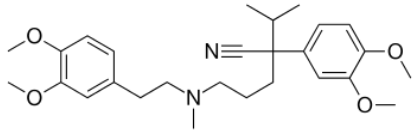
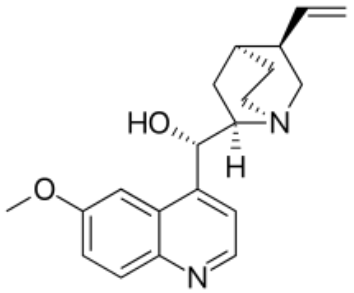
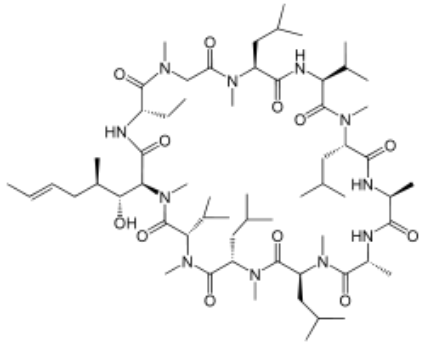
P-gp Inhibitors		Structure	Therapeutic Drugs	Inhibitor of			Representative P-gp Assays
				P-gp	BCRP	MRP	
First Generation	Verapamil		Cardiac / circulation Drugs	√[1]	√[1]	x[2]	Increased Rho123 accumulation in K562Dox cell line [3] Increased ATPase activity (Competitive P-gp inhibitor) [3] 5.9-fold decrease in doxorubicin GI50 K562Dox cell line [3]
	Quinidine		Antimalarial Drugs	√[4]	√[4]		Weak effect on doxorubicin accumulation in a resistant human erythroleukemia cell line [5] Completely restored doxorubicin sensitivity in the resistant human erythroleukemia cell line [6]
	Cyclosporine A		Immunossu-ressant Drugs	√[7]	√[8]	√[8]	Combination with doxorubicin on K562/A02 cells: MDR partially reversed [9] Combination with doxorubicin on hepatocellular carcinoma cell lines: decreased doxorubicin IC50 [10] Photo affinity labelling with [^{3}H]azidopine: P-gp competitive inhibition [11]

Table 2.4 *Continued*

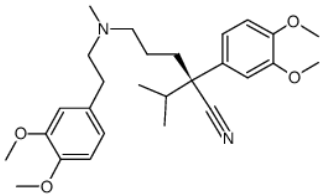
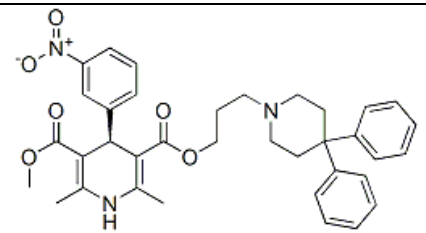
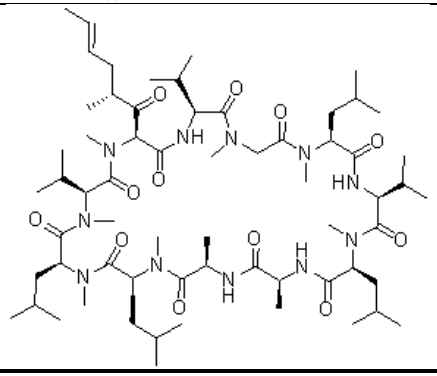
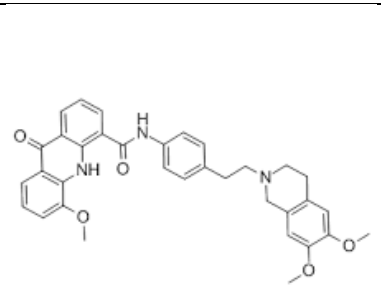
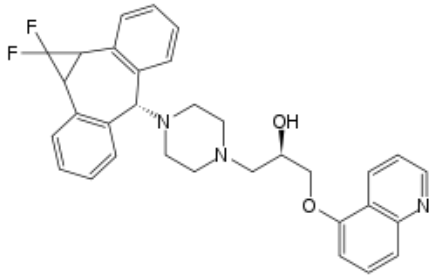
P-gp Inhibitors		Structure	Derivative/ Stereo - isomer of	Inhibitor of			Representative P-gp Assays
				P-gp	BCRP	MRP	
Second Generation	Dexverapamil		verapamil	√[1]	x[1]		Increased [^{3}H]vimblastine accumulation in theF4-6RADR cell line [12] Combination of dexverapamil with DINIB, a P-gp substrate, in the colon cancer cell line, HCT-15, and renal cell line, UO-31: reversed P-gp-mediated resistance in both cell lines, increasing DINIB cytotoxicity [13]
	Dexniguldipine		niguldipine	√[12]			Reversed vinblastin resistance in F4-6RADR cells [12]. Decreased adriamycin GI50 in adriamycin resistant erythroleukemia F4-6RADR cells [14]
	Valspodar (PSC 833)		cyclosporine	√[15]	x[15]	√[15]	Decreased doxorubicin GI50 in SK-MES-1/DX1000 resistant cells, but also downregulated P-gp expression by activating JNK/c-Jun/AP-1 and suppressing NF-κB [16] Administration of valspodar to rats before mitoxantrone treatment: increased the accumulation of mitoxantrone in the MDR tumors to 94% of that in the wild-type tumors [17]

Table 2.4 *Continued*

P-gp Inhibitors		Structure	Inhibitor of			Representative P-gp Assays
			P-gp	BCRP	MRP	
Third Generation	Elacridar (GF120918)		√[18]	x[19]	√[19]	Increase verapamil distribution in rat brain (which expresses P-gp) up to 11-fold over baseline at maximum effective doses, with elacridar being about three times more potent than tariquidar [20] Combination with etoposide, doxorubicin, vinblastine, docetaxel and paclitaxel in MDR sarcoma MES-Dx5 cells: reversal of resistance [21] Significant increase of the systemic exposure of topotecan, leading to an increase of oral bioavailability [22]
	Zosuquidar (LY335979)		√[23]	x[23]	x[23]	Increased paclitaxel levels in plasma and tissues in mice to levels similar to those observed in P-gp knockout mice [24] Combination with imatinib in mice: improved the delivery of imatinib to the brain, making it potentially more effective against malignant gliomas [25]

√ stands for the substrates. × stands for this drug is neither inhibitor nor substrates for the transporter.

[1] (Perrotton et al., 2007) [2] (Leibach and Ganapathy, 1996) (Leibach and Ganapathy, 1996) (Leibach and Ganapathy, 1996) (Leibach and Ganapathy, 1996) (Leibach and Ganapathy, 1996) (Leibach and Ganapathy, 1996) (Leibach and Ganapathy, 1996) [3] (Palmeira et al., 2012) [4] (Vezmar and Georges, 2000) [5] (Lehnert et al., 1991) [6] (Bennis et al., 1995) [7] (Pawarode et al., 2007) [8] (Gupta et al., 2006) [9] (Chen et al., 2008) [10] (Shiraga et al., 2001) [11] (Ueda et al., 1994) [12] (Hollt et al., 1992) [13] (Bates et al., 2004) [14] (Dietel et al., 1996) [15] (Breedveld et al., 2006) [16] (Bark and Choi, 2010) [17] (Shen et al., 2009) [18] (Stokvis et al., 2004) [19] (Lagas et al., 2009) [20] (Kuntner et al., 2010) [21] (Traunecker et al., 1999) [22] (Kruijtzer et al., 2002) [23] (Shepard et al., 2003) [24] (Kemper et al., 2004) [25] (Bihorel et al., 2007)

2.1.1 First Generation

2.1.1.1 Verapamil

In 1981, the first description of verapamil was made as a potential MDR reversing agent, indicating the possibility of identifying clinically useful reversing agents of MDR (Tsuruo and Fidler, 1981). The calcium channel blocker, verapamil, was the first compound ever found which was able to enhance the intracellular accumulation of many anticancer drugs such as vincristine, and doxorubicin (Bellamy et al., 1988, Yusa and Tsuruo, 1989). Indeed, it was demonstrated that verapamil inhibited the efflux of anticancer drugs from tumor cells that over-expressed P-gp, causing an increase in the intracellular concentration of the chemotherapeutic drug. Some authors suggest that verapamil inhibits P-gp activity by direct competition with P-gp substrates (Futscher et al., 1996).

2.1.1.2 Cyclosporine A

In 1986, an immunosuppressive drug, cyclosporine A (CsA), demonstrated that it also had the capacity to reverse resistance to anticancer drugs *in vitro* (Slater et al., 1986). CsA was reported to interfere with the P-gp mediated reaction. The truth is, it has been demonstrated that CsA competed with the substrates of P-gp to bind to the drug-binding site of P-gp (Sakata et al., 1994). At the beginning of the 90s, the first clinical trial with CsA and anticancer drugs were initiated in patients with multiple myeloma and acute leukemia (Bartlett et al., 1994, List et al., 1993, Yahanda et al., 1992). Subsequently, several other clinical trials were performed. However, CsA showed no selectivity towards P-gp. It is able to increase cellular drug uptake in cells overexpressing P-gp, MRP-1 or BCRP and nuclear drug uptake in cells overexpressing LRP (Gupta et al., 2006, Pawarode et al., 2007).

2.1.2 Second Generation

Owing to the experience with the first-generation compounds, the approach subsequently followed was to identify analogues that were devoid of the pharmacological properties of the original molecule but could specifically inhibit P-gp, with less toxicity and a greater potency (Krishna and Mayer, 2000).

2.1.2.1 Valspodar (PSC 833)

Valspodar was developed by Novartis and derives from CsA due to a methylation in a lateral chain of an amino acid and an oxidation of an alcohol to a carbonyl. It is a nonimmunosuppressive cyclosporine analog which is a potent MDR modifier (5- to 20-fold more potent than CsA) (Boesch et al., 1991, Kusunoki et al., 1998). The main problem associated with this compound is the interaction with the pharmacokinetics of the associate chemotherapeutic drugs, which resulted to an increase in the chemotherapeutic drug toxicity, sequentially requiring a reduction of its dose (Chico et al., 2001).

2.1.3 Third Generation

To overcome the limitations of the second generation P-gp modulators, a third-generation of P-gp inhibitors, which specifically and potently inhibit P-gp, has been developed by using quantitative structure-activity relationships (QSAR) and combinatorial chemistry (Krishna and Mayer, 2000).

2.1.3.1 Tariquidar (XR9576)

Tariquidar (XR9576) is an anthranilamide derivative and an example of a third generation P-gp inhibitor (Fox and Bates, 2007) and has long been described as a specific P-gp inhibitor. However, it is now accepted that tariquidar (Kelly et al., 2011) and elacridar (Lagas et al., 2009) also bind with breast cancer resistance protein (BCRP). Tariquidar binds P-gp through a noncompetitive mechanism and with an affinity that greatly exceeds that of the transported substrates (Bauer et al., 2013). It inhibits the ATPase activity of P-gp, however, it is not clear whether the binding of tariquidar on P-gp is directed to the ATP binding site or to an allosteric location, thus, indirectly blocking the P-gp catalytic cycle (Martin and Baxter, 1999).

Various types of compounds and techniques for the reversal of P-gp-mediated MDR have been developed and the strategy has been mainly to inhibit the function of the pump. It has been 35 years since the discovery of the first P-gp inhibitor and 48 years since the isolation of the first MDR cells. However, some defeatism still remains on the possibility of finding a “perfect” P-gp inhibitor that can efficiently modulate the pump and restore the efficacy of chemotherapy. Considering the purpose of this study and the cost of P-gp inhibitors into account, the first generation P-gp inhibitor, cyclosporine A (CsA), was chosen to start a comprehensive analysis of the efficacy of P-gp inhibitors in this sex-based phenomenon in Phase II.

Phase I

2.2 MATERIALS AND METHODS

2.2.1 Materials

Fexofenadine hydrochloride was purchased from Cambridge Bioscience Ltd. (Cambridge, UK). Metformin hydrochloride and ampicillin sodium was obtained from USV Ltd. (Mumbai, India) and VWR International (Lutterworth, UK), respectively. Polyethylene glycol 400, sodium dodecyl sulfonate and HPLC-grade water were supplied by Sigma-Aldrich (Dorset, UK). HPLC-grade reagents such as acetonitrile, methanol and glacial acetic acid were obtained from Fisher Scientific (Loughborough, UK). Analytical grade reagents such as ammomium acetate and sodium dihydrogen phosphate were procured from VWR International (Lutterworth, UK).

2.2.2 Animals

All the animal work was approved by the UCL School of Pharmacy's ethical review committee and was conducted in accordance with the home office standards under the Animals (Scientific Procedures) Act, 1986. Healthy male and female, 8-13 week old Wistar rats (Harlan UK Ltd, Oxfordshire, UK) weighing 150-250 g were used. The rats were housed at controlled temperatures (25℃) and humidity (50-60%) with a constant light-dark cycle of 12h. Food and water were provided and the rats were acclimatized for 7 days before being studied.

2.2.3 Dose studies on the model drugs

Fexofenadine, ampicillin and metformin were weighed accurately and dissolved with water, prepared in 10mg/mL, 15mg/mL and 25mg/mL. Only male Wistar rats were used for these dose studies.

Rats were divided into three groups and administered with 0.5mL solutions in different concentrations. After dosing, approximately 250μL-300μL of blood was collected from the tail vein of rats into anticoagulant centrifuge tubes (BD Microtainer® K2E Becton, Dickinson and Company, USA) at 0.5, 1.25, 2, 3, 4 and 6h. At 8h post-administration, the rats were sacrificed in a CO_2 euthanasia chamber and about 1mL of blood was taken via cardiac puncture immediately.

2.2.4 Effect of PEG 400 on drug bioavailability

The day before the experiment, all the rats were fasted overnight and individually housed in metabolic cages.

On the day of the experiment, each rat was weighed and administered an aqueous solution, corresponding to a dose of fexofenadine, ampicillin or metformin (selected based on the results from section 2.3.3) with or without 26mg/kg PEG 400 by oral gavage. PEG 400 was used at a dose of 26mg/kg as this caused the greatest enhancement in ranitidine bioavailability in rats (Afonso-Pereira et al., 2016). Subsequently, approximately 250μL-300μL of blood was collected from the rats' tail vein into anticoagulant centrifuge tubes (BD Microtainer® K2E Becton, Dickinson and Company, USA) at 0.5, 1.25, 2, 3, 4 and 6h. At 8h post-administration, the rats were killed in a CO_2 euthanasia chamber and about 1mL of blood was taken by cardiac puncture.

2.2.5 Samples preparation

Blood samples were centrifuged at 10,000rpm for 10min, and the supernatants (plasma samples) were collected into 1.5mL Eppendorf tubes.

Fexofenadine was extracted from a 100μL plasma sample by protein precipitation with 300μL acetonitrile. The mixture was vortex-mixed for 6s and centrifuged at 13,000rpm for 15min. The supernatant was then collected and evaporated to dryness in a Savant SPD2010 SpeedVac Concentrator system (Thermofisher, 55303 Minnesota, USA). The dried residue was dissolved in a 100μL diluent (a mixture of methanol-acetate buffer in the ratio of 50:50) and vortex-mixed for 30s. An aliquot of 50μL of each sample was then processed on the HPLC apparatus.

For ampicillin and metformin, 100μL of acetonitrile or methanol was added to 100μL of plasma samples respectively, to precipitate the protein. The mixtures were vortex-mixed for 6s and centrifuged in a Centrifuge 5804R (Eppendorf AG, 22331 Hamburg, Germany) at 13,000rpm for 10min. The supernatant was then collected and a 50μL aliquot of each sample was analysed by HPLC.

2.2.6 Chromatographic analysis

The amount of drug in the withdrawn samples was quantified using a high performance liquid chromatography (HPLC) system (Agilent Technologies, 1260 Infinity) equipped with a pump (model G1311C), autosampler (model G1329B) and a diode-array UV detector (model G1314B). All the peaks of model drugs can be achieved with a Luna C18(2) 100A (250mm×4.6mm I.D./5μm particle size, Phenomenex, Torrance, USA). The best suited HPLC conditions for quantification of four model drugs in rat plasma were shown in Table 2.5

Table 2.5 Chromatographic conditions and parameters for quantification of fexofenadine, ampicillin and metformin in rat plasma.

Model Drugs	Column	Column Temperature	Detector	Wavelength	Mobile Phase	Flow Rate	Injection Volume
Fexofenadine	C18 (250mm×4.6mm I.D./5μm)	25℃	Fluorescence	Excitation: 230nm Emission: 310nm	0.1M Ammonium Acetate Buffer (pH 4.0, 63%); Acetonitrile (37%)	1mL/min	50μL
Ampicillin	C18 (250mm×4.6mm I.D./5μm)	25℃	Ultraviolet	220nm	10mM Sodium Dihydrogen Phosphate Buffer (pH 7.0, 60%); Methanol (40%)	0.6mL/min	50μL
Metformin	C18 (250mm×4.6mm I.D./5μm)	25℃	Ultraviolet	234nm	10mM Sodium Dihydrogen Phosphate Buffer with 10mM Sodium Dodecyl Sulfonate (pH 7.0, 60%); Acetonitrile (40%)	1mL/min	50μL

2.2.7 HPLC method validation

Quality control standards of low, medium and high concentrations of the molecules were prepared in control rat plasma to evaluate the precision and accuracy of the method. Separate standards of low concentrations were prepared to investigate the limit of detection and quantification.

2.2.7.1 Linearity

Calibration curves are constructed daily, for five consecutive days. The linearity of the methods was evaluated with a total of seven calibration standards over the concentration range 20-1000ng/mL for fexofenadine, 0.2-20μg/mL for ampicillin and 50-5000ng/mL for metformin. Coefficients of calibration equation and the correlation coefficient were expressed.

2.2.7.2 Detection and quantification limits (sensitivity)

According to the visual evaluation method, limit of detection (LOD) is determined by establishing the minimum level at which the analyte can be reliably detected. Limit of quantitation (LOQ) is considered as the lowest concentration of analyte in standards that can be reproducibly measured with an acceptable accuracy of 80-120% and precision ≦20%.

2.2.7.3 Assay precision and accuracy

Accuracy, intra- and inter-day precisions of the method were determined for three model drugs according to FDA guidance. The quality control samples (low, medium and high) were investigated using five determinations on the same day. The inter-day variation was also evaluated at these three different concentrations on five different non-consecutive days.

The accuracy was calculated from the mean value of observed concentration (Ca) and the nominal concentration (C_b) as follows: Accuracy(%)=[(C_a-C_b)/C_b]×100.

The precision coefficient of variation, C.V. (%) was calculated from observed concentrations as follows: Precision, C.V.(%)=[standard deviation(S.D.)/C_a]×100.

2.2.7.4 Recovery

Three quality control samples were prepared in diluents and blank rat plasma. The mean peak areas were then collected. Recovery was calculated by comparing the mean peak area of an extracted sample (n=5) to the one obtained after the direct injection of a solution with the same drug concentration.

2.2.7.5 Stability tests

2.2.7.5.1 Untreated plasma short-term stability

The quality control samples were maintained at ambient temperature for 2h. The model drugs in the samples were then processed following the HPLC method. The stability was calculated by comparing the mean peak area of samples treated immediately and the untreated samples.

2.2.7.5.2 Post-preparative short-term stability

Six plasma samples in three different concentrations were prepared. Three of them were investigated with HPLC immediately, whist the other three were processed 24h later. The stability was calculated as follows:

$$\text{Stability (\%)} = \frac{\text{Peak area response of samples at 0h}}{\text{peak area response of samples at 24h}} \times 100$$

2.2.7.5.3 Repetitive freeze-thaw stability

Repetitive freeze-thaw stability of the quality control samples of model drugs in rat plasma samples was determined for three freeze-thaw cycles. The samples were thawed at room temperature, and then kept in the freezer at -20℃ for 24h before the next thawing.

2.2.7.5.4 Long-term stability

The quality control samples were assessed by performing the experiment after 30 days of storage at -20℃. The stability was calculated from the relative recovery as follows:

$$\text{Recovery (\%)} = \frac{\text{Peak area response of samples on 0 day}}{\text{Peak area response of samples on 30 days}} \times 100$$

2.2.8 Pharmacokinetic analysis

Pharmacokinetic parameters, (C_{max}, t_{max}, $AUC_{0\text{-}480}$, AUC_{∞}, CL, Vd and $t_{1/2}$) were calculated by non-compartmental analyses using a free Microsoft Excel add-in, "PKSolver." (Zhang et al., 2010).

2.2.9 Statistical analysis

All results are expressed as mean ± SD (n = 6). The control and test group data were analysed by one-way ANOVA, followed by post-hoc Tukey analysis with a 95 % confidence interval using IBM SPSS Statistics 16 (SPSS Inc., Illinois, USA). Repeated measures ANOVA was conducted to assess any statistically significant differences along the absorption profiles.

2.3 RESULTS AND DISCUSSION

2.3.1 HPLC method validation

2.3.1.1 Optimization of chromatographic conditions

Chromatographic conditions were optimized by changing the mobile phase composition and buffers used in the mobile phase.

For fexofenadine, the mixture of 0.05M phosphate buffer and methanol in the ratio 65:35(v/v) was used as mobile phase in the beginning, however, the peak area of fexofenadine was small and the limit of detection (LOD) was high. The mobile phase consisted of 63% 0.1M acetate buffer. 37% acetonitrile was then use which achieved optimized results. By altering the pH of mobile phase, better separation of target drug from plasma was accomplished. Various excitation and emission wavelengths have also been used to determine fexofenadine in rat plasma, to be specific, a 5^2 factorial design was supplied to obtain the best peak area. The selection of levels for the optimization was based on the wavelengths have been used in other experiments, and the largest peak area have been found when the fluorescence measurements for fexofenadine were done at 230nm excitation and 310nm emission wavelengths. Typical chromatograms using the HPLC method with fluorescence detection for blank rat plasma and fexofenadine in plasma at the medium are shown in Figure 2.1. The retention time of fexofenadine was 8.6min.

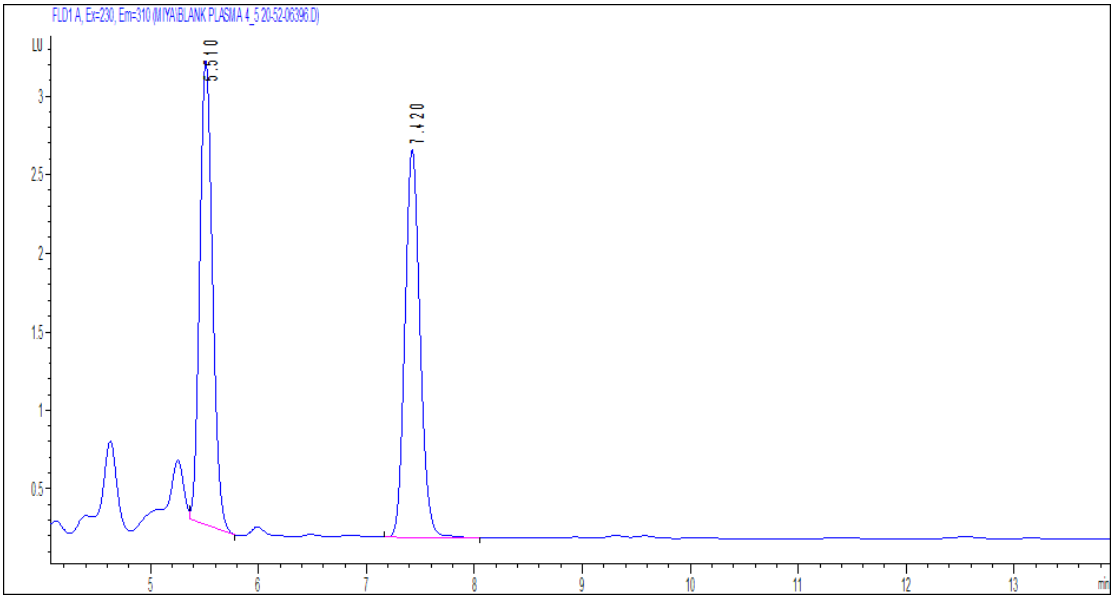
For ampicillin, the LOD was 5μg/mL in rat plasma when 60% 0.1M sodium dihydrogen phosphate and 40% acetonitrile was used as the mobile phase, which was not suitable in rat plasma. Following this procedure, the organic phase was changed to acetonitrile instead of methanol where the LOD decreased to 0.1μg/mL. However, the column pressure was increases from 220bar to 240bar. To modify the method, the flow rate was reduced from 1mL/min to 0.6mL/min which helped ease the column pressure to 190bar. Chromatogram profiles of ampicillin were obtained in Figure 2.2.

For metformin, the retention time was too early to detect the drug in rat plasma when 60% 10mM phosphate buffer and 40% acetonitrile was used as mobile phase. As it is reported that there are complex reactions between sodium dodecyl sulfonate and phosphate radical, the retention time was successfully delayed to 12 min by adding 10mM sodium dodecyl sulfonate in the phosphate buffer. However, when the sodium dodecyl sulfonate and potassium dihydrogen phosphate buffer were mixed, potassium precipitated out from the mixture and white floccule could be found in the solution. Therefore, 10mM sodium dihydrogen phosphate was used instead of potassium dihydrogen phosphate buffer. Typical chromatograms of blank rat plasma and metformin in plasma at the medium were shown in Figure 2.3.

2.3.1.2 Linearity

Calibration curves for fexofenadine, ampicillin and metformin in rat plasma were linear over the concentration range of 20-1000ng/mL, 0.2-20μg/mL and 50-5000ng/mL, respectively. Typical calibration curves, which were analyzed for five different days respectively, could be expressed for plasma samples as $y=(0.0041\pm0.0003)x+(0.1653\pm0.0836)$, $y=(36.2490\pm1.2347)x-(0.2516\pm1.9454)$ and $y=(0.1263\pm0.0033)x-(0.5374\pm1.8189)$, where y is the peak area and x is the concentration in ng/mL with a mean correlation coefficient of 0.9977, 0.9999 and 0.9998.

A



B

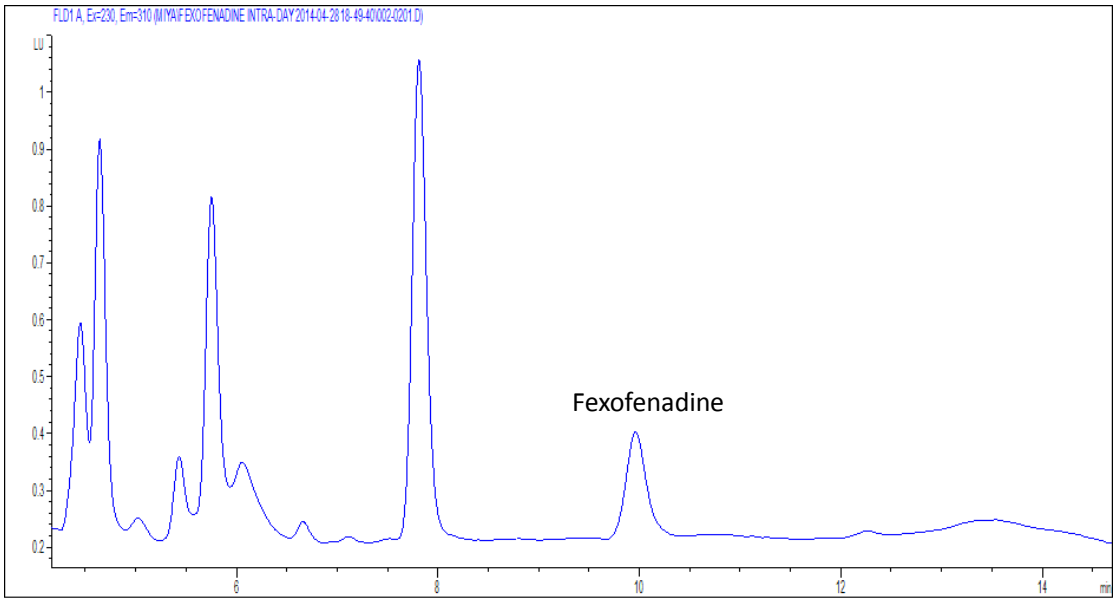
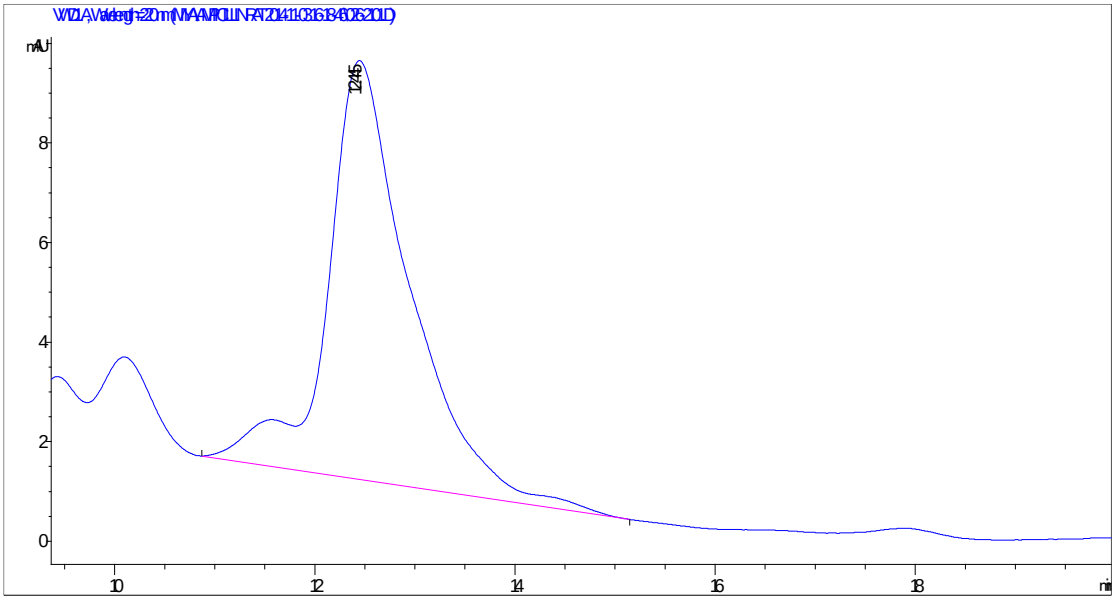


Figure 2.1 Representative chromatograms of (A) blank rat plasma and (B) blank rat plasma spiked with 150ng/mL fexofenadine.

A



B

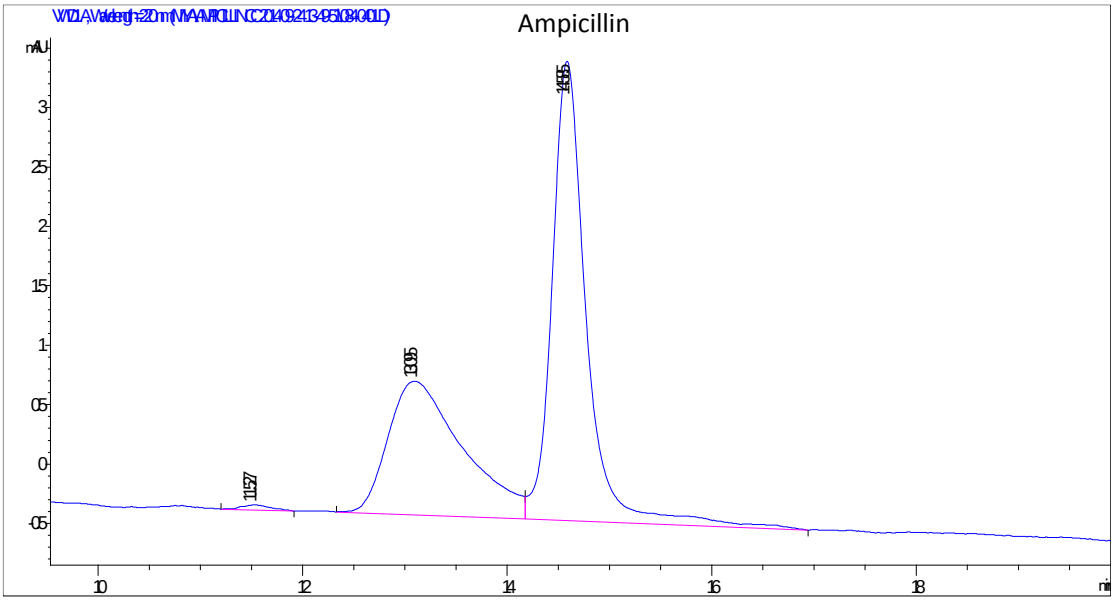
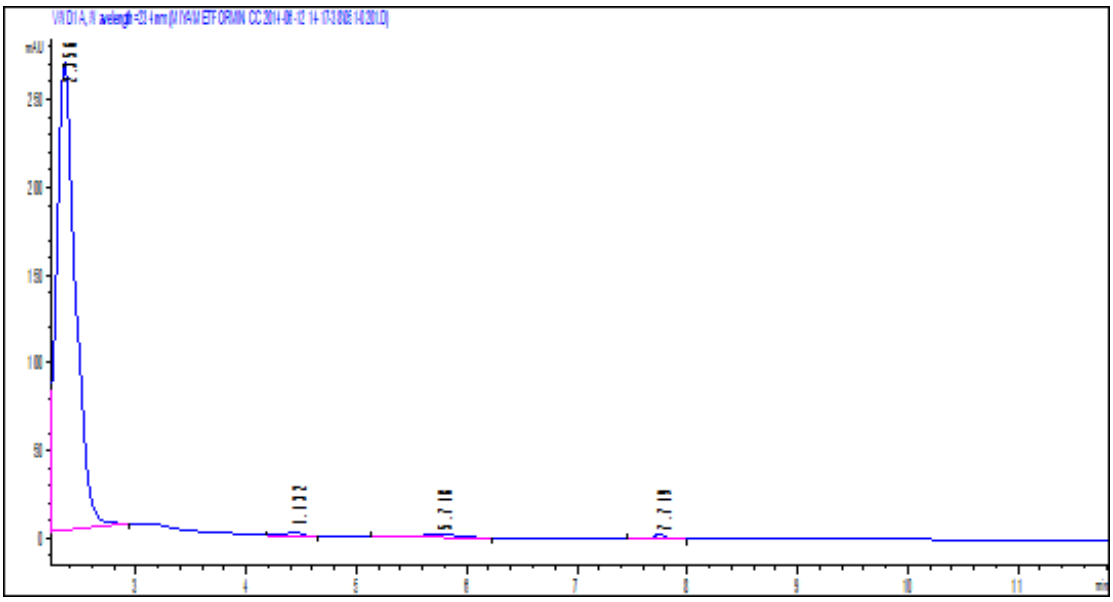


Figure 2.2 Representative chromatograms of (A) blank rat plasma and (B) blank rat plasma spiked with 2µg/mL ampicillin.

A



B

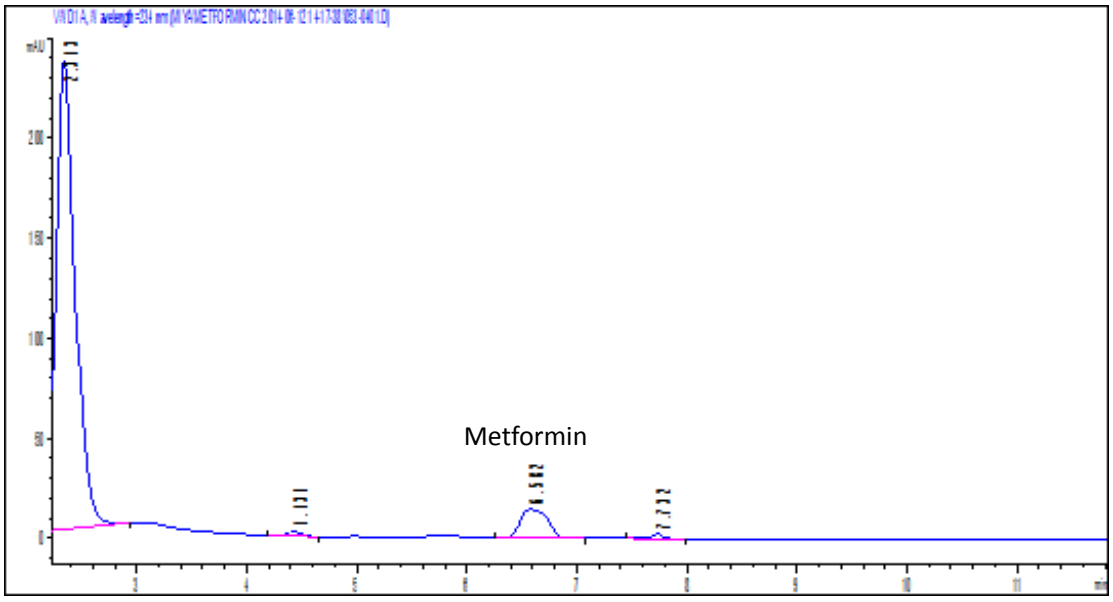


Figure 2.3 Representative chromatograms of (A) blank rat plasma and (B) blank rat plasma spiked with 2µg/mL metformin.

2.3.1.3 Limit of detection and limit of quantitation (sensitivity)

The lower limit of quantification (LOQ) of fexofenadine, ampicillin and metformin were found to be 10ng/mL, 200ng/mL and 20ng/mL, respectively. While the accuracy (%bias) for these three model drugs were round 92.34%, 90.15% and 98.23%, with a precision (C.V.) not exceeding 20%. The limit of detection (LOD) considering a signal-to-noise ratio 3:1 were estimated to be 5ng/mL, 100ng/mL and 20ng/mL for fexofenadine, ampicillin and metformin respectively.

2.3.1.4 Accuracy and precision

The accuracy and precision of the method were evaluated with the samples at three different concentrations. The intra- and inter-day accuracy and precision values of the assay method are shown in Table 2.6.

The intra-day C.V. (%) for fexofenadine was below 19.03%, while they were below 1.48% and 1.63% for ampicillin and metformin respectively. All inter-day C.V. (%) for drugs were below 6.17%. The intra-day and inter-day accuracies for fexofenadine were found to be within 96.55% and 112.33%, while these two parameters for ampicillin were not above 98.56% and 105.59%, of the target values. For metformin, the accuracies were observed between 96.16% and 109.97%.

Table 2.6 Intra- and inter-day accuracy and precision of fexofenadine, ampicillin and metformin determination at three concentration levels (n = 5).

Samples (ng/mL)	Measured Concentration (ng/mL)	Accuracy (%)	Intra-Precision C.V. (%)	Inter-Precision C.V. (%)
Fexofenadine				
20	22.47±4.27	112.33	19.03	4.06
150	158.02±4.27	105.35	2.71	2.30
1000	979.80±12.17	97.98	1.24	1.50
Ampicillin				
500	527.96±4.21	105.59	0.80	6.17
2,000	2045.30±26.77	102.26	1.31	1.84
20,000	20659.61±306.48	103.30	1.48	0.94
Metformin				
100	109.97±1.31	109.97	1.19	2.43
2000	1923.12±27.93	96.16	1.45	2.81
5000	5007.15±76.99	101.25	1.63	1.54

2.3.1.5 Recovery

The mean extraction recovery of fexofenadine at the three different concentrations were 92.52%, 98.35% and 99.19% respectively, whilst metformin showed a better recovery (96.88%, 97.43% and 99.46%). The ampicillin recovery from rat plasma were not as high as those of fexofenadine and metformin, shown in Table 2.7.

Table 2.7 Recovery of fexofenadine, ampicillin and metformin in plasma.

Sample (ng/mL)	Concentration(ng/mL)				Mean	S.D.	Recovery (%)
	Add	Found					
Fexofenadine							
20	19.80	17.58	19.80	17.58	18.32	1.28	92.52
150	147.49	142.61	145.05	147.49	145.05	2.44	98.35
1000	1008.46	984.07	1032.85	984.07	1000.33	28.16	99.19
Ampicillin							
500	537.61	490.51	484.84	504.70	494.35	10.23	91.77
2,000	2051.82	1909.11	1954.51	1920.46	1928.03	23.62	93.97
20,000	20830.41	18909.62	18248.56	18745.07	18634.42	344.14	89.46
Metformin							
100	113.73	111.22	109.66	109.66	110.18	0.90	96.88
2000	1989.39	1953.94	1942.21	1918.73	1938.29	17.93	97.43
5000	4956.14	4978.98	4874.91	4933.60	4929.17	52.18	99.46

2.3.1.6 Stability

The stability of fexofenadine, ampicillin and metformin in plasma were investigated in four different studies using samples at three concentration levels. The results were showed in Table 2.8.

For fexofenadine and metformin, the stability of plasma samples at room temperature was examined by comparing the data of the samples analyzed immediately with those at 2h after sample preparation, and the results showed no significant change in the concentration of all the model drugs. The 24h autosampler stability results of fexofenadine in plasma ranged from 91.67% to 98.13%, which showed a good stability. Better stability was found for metformin with the recovery from 92.13% to 101.56%. The freeze and thaw cycles did not change the concentration levels of analyte significantly, whereas the long-term data showed that both fexofenadine and metformin were stable for 30 days in rat plasma too.

For ampicillin, the stability of the samples during room temperature and during their storage at 4°C was also analyzed. No significant change in peak area was observed if the samples were stored at 4°C, where at least 94.79% and 89.86% recovery was observed in short-term and long-term stability studies. However, if the samples were allowed to sit at room temperature, there was, at least, a 10% increase in the peak area after 2h, as determined by the HPLC assay. Lowest stability was found after freeze and thaw cycles, which was only 81.23% recovery for the low concentration sample.

Table 2.8 Stability of fexofenadine, ampicillin and metformin in rat plasma at three concentration levels (n=3).

Samples (ng/mL)	Stability (%)			
	Untreated Plasma Short-Term Test	Post-Preparative Short-Term Test	Freeze-Thaw Test	Long-Term Test
Fexofenadine				
20	93.55	91.67	92.00	96.43
150	95.56	98.73	85.43	84.55
1000	91.30	95.24	93.62	92.00
Ampicillin				
500	82.83	94.79	81.23	91.03
2,000	87.27	97.60	82.66	90.96
20,000	88.07	96.05	85.74	89.86
Metformin				
100	105.79	92.13	92.40	98.80
2000	99.76	97.89	91.68	98.21
5000	99.32	101.56	100.03	99.78

2.3.2 Dose studies

2.3.2.1 Fexofenadine

From the data shown in Figure 2.4, no extreme differences were identified in the drug plasma concentration when fexofenadine was administered at 10mg/kg, 20mg/kg and 50mg/kg. However, in the 10mg/kg administration group, the detected fexofenadine concentration was only 3ng/mL at 8h, which was below 5ng/mL (LOD). Therefore, 20mg/kg or 50mg/kg fexofenadine could be given to the rats in the bioavailability studies.

It was previously reported that 10mg/kg fexofenadine was used in the rat studies. However, the pharmacokinetic studies of fexofenadine in rats have shown a relatively low bioavailability when administered orally. For example, a single oral dose of 10 mg/kg yielded the highest plasma level of 20-25ng/mL. Although a HPLC method with fluorescence detection has been developed, and the LOD is 5ng/mL, it would be better to attempt a high dose in the pharmacokinetic studies.

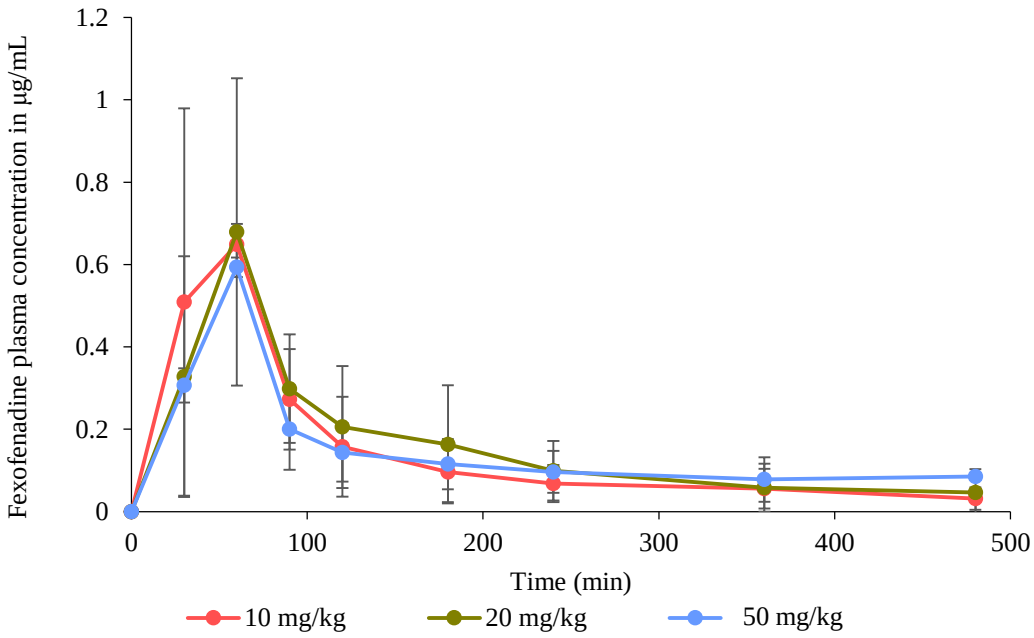


Figure 2.4 Concentration-time curve of fexofenadine in male rat plasma in dose study (Mean ± S.D., n=3).

2.3.2.2 Ampicillin

Three different concentrations of ampicillin (20mg/kg, 30mg/kg and 50mg/kg) were prepared in water and administered to rats. However, the ampicillin plasma concentration was too low to be detected with our HPLC method at some time points, for example, when the rats were given 20mg/kg or 30mg/kg ampicillin (shown in Figure 2.5). 50mg/kg ampicillin was therefore administered during the rat experiments.

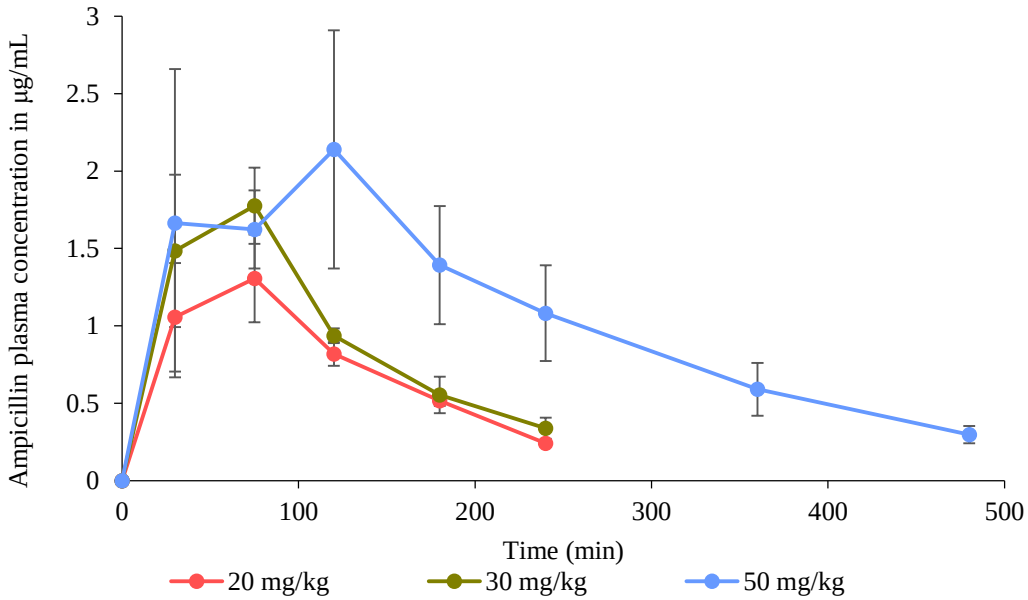


Figure 2.5 Concentration-time curve of ampicillin in male rat plasma in dose study (Mean ± S.D., n=3).

2.3.2.3 Metformin

High doses (50mg/kg, 100mg/kg, 200mg/kg) were used in the former pharmacokinetic studies of metformin in rats. However, the last blood sample was collected on 48h. In our studies, we collected seven samples over 8 hours, therefore, lower concentrations of metformin were utilized in the dose studies. Concentrations of metformin in rat plasma were significantly different when this compound was administered in different concentrations, outlined in Figure 2.6.

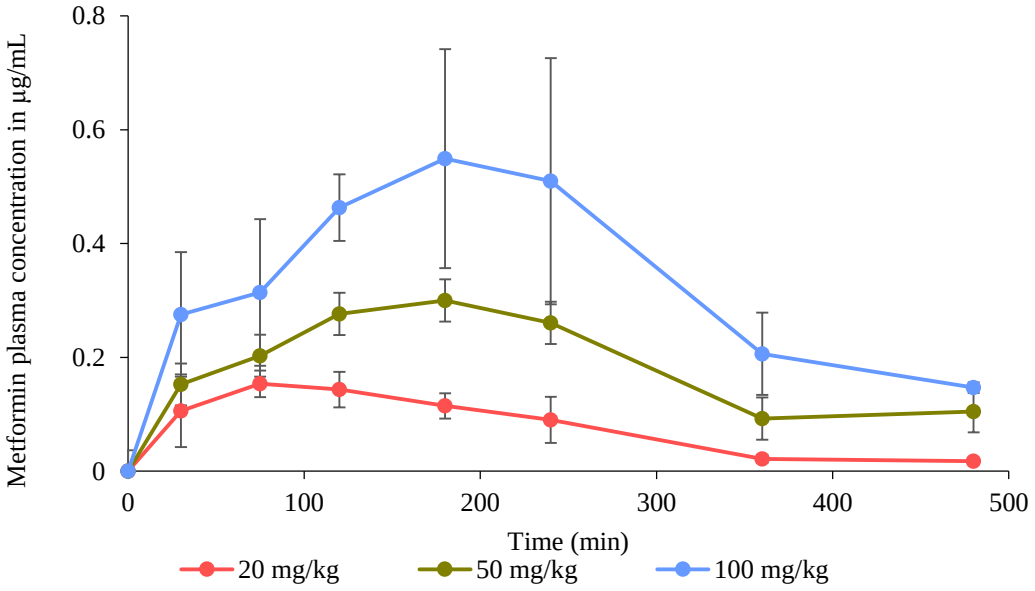


Figure 2.6 Concentration-time curve of metformin in male rat plasma in dose study (Mean ± S.D., n=3).

In short, considering 50mg/kg ranitidine was used in the previous study and 50mg/kg ampicillin was selected to be given to the rats in this study, same dose of fexofenadine and metformin were utilized in the bioavailability study with the aim of minimizing the variable factors.

2.3.3 Effect of PEG 400 on drug bioavailability

2.3.3.1 Fexofenadine

The bioavailability of fexofenadine in the absence and presence of PEG 400 are observed in Figure 2.7, Figure 2.8 and Table 2.9. It stands that the effect of PEG 400 on the pharmacokinetic parameters of fexofenadine were significant in both male and female rats ($p<0.05$ for C_{max} and AUC), however, a greater impact was found in male rats compared with females.

To be specific, the fexofenadine AUC_{0-480} for both male and female control group was 13.8±3.1μg.min/mL and 22.6±7.3μg.min/mL respectively. The presence of PEG 400 enhanced the bioavailability of fexofenadine to 55.6±13.5μg.min/mL and 62.48±10.0μg.min/mL in males and females, ($p<0.05$). However, a greater response was found in the effect of PEG 400 on the bioavailability of fexofenadine in males (an increase with 150%) when compared with female rats in which the induction was only approximately 93%.

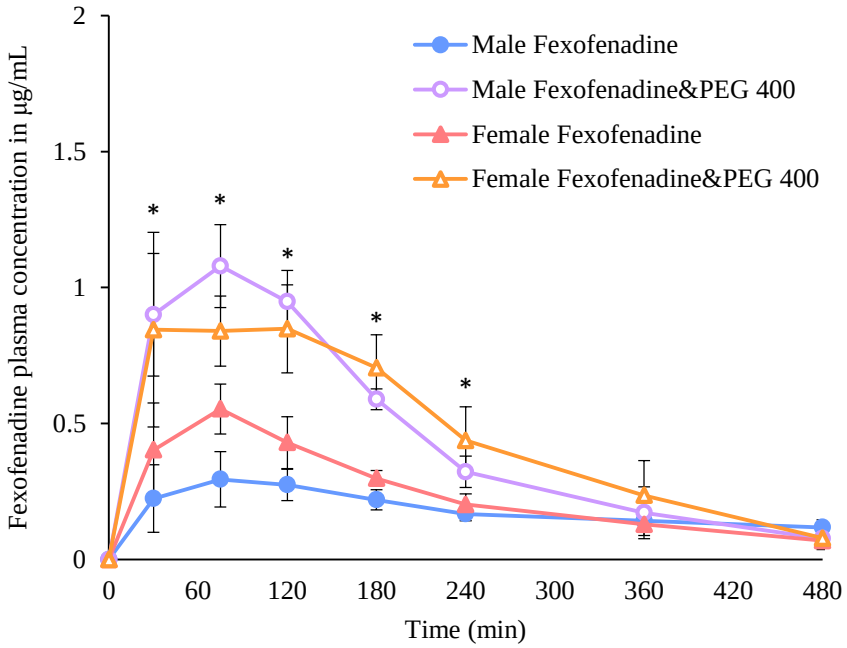


Figure 2.7 Plasma concentration-time profile of fexofenadine in the absence and presence of PEG 400 over 8 hours in male and female Wistar rats (Mean ± S.D., n=6).* Value is statistically different at $p<0.05$.

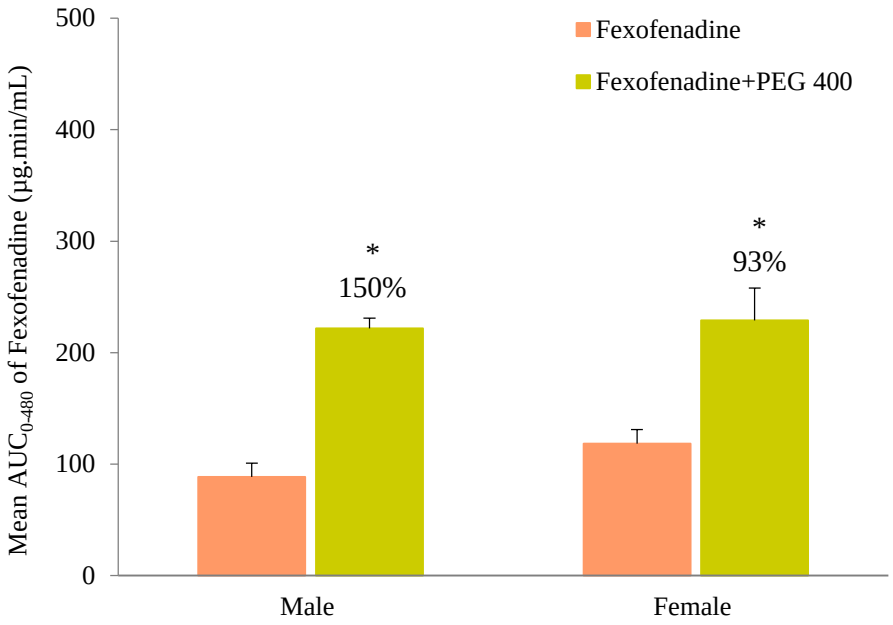


Figure 2.8 Mean $AUC_{0\text{-}480}$ of fexofenadine in the presence and absence of PEG 400 in male and female Wistar rats (Mean ± S.D., n=6). * Values are statistically different between the control and PEG groups at $p<0.05$.

Table 2.9 Influence of 26mg/kg PEG 400 on the pharmacokinetic parameters of fexofenadine in male and female rats (Mean±S.D., n=6).

Pharmacokinetic Parameters	**Male**		**Female**	
	Control	**PEG 400**	**Control**	**PEG 400**
$AUC_{0\text{-}480}$ ($\mu g.min/mL$)	88.62±12.30	221.78±9.11 *	118.45±12.62	228.83±29.19*
AUC_{∞} ($\mu g.min/mL$)	132±18.40	234.64±11.18*	135.24±20.59	244.97±30.57*
c_{max} ($\mu g/mL$)	0.34±0.06	1.14±0.07*	0.58±0.07	1.08±0.09*
t_{max} (min)	67.50±33.88	75.00±28.46*	60.00±23.23	67.50±44.24
CL (mL/min)	0.07±0.04	0.05±0.003	0.09±0.01	0.05±0.006
Vd (mL)	50.69±18.51	8.64±0.95*	21.04±2.84	9.84±2.08*
$t_{1/2}$ (min)	299.22±76.35	112.74±16.12*	156.94±27.45	133.91±36.06

* Values are statistically different between the control and PEG groups at $p<0.05$.

The findings in the case of fexofenadine was partly in conflict with our hypothesis which stated that PEG 400 showed sex-related impact on the bioavailability of P-gp substrates, since fexofenadine was confirmed as a typical substrate for the efflux transporter P-gp alongside the influx transporter OATP1A2. However, a recent study demonstrated that PEG 400 was not only a selective modulator of P-gp but also OATP1A2 (Engel et al., 2012), which may make the result too complicated to be analyzed. Therefore, the bioavailability of another P-gp substrate, ampicillin with PEG 400, need to be investigated in male and female rats, as followed.

2.3.3.2 Ampicillin

The influences of PEG 400 on ampicillin absorption in male and female rats are shown in Figure 2.9, Figure 2.10 and Table 2.10. It can be seen that ampicillin absorption profiles and pharmacokinetic parameters were similar for males and females in the absence of PEG 400 ($p>0.05$). The presence of PEG 400 caused no changes in the drug absorption in females ($p>0.05$). In contrast, PEG 400 had a considerable influence on ampicillin absorption in male rats ($p<0.05$ for t_{max}, C_{max} and AUC), where ampicillin absorption was more rapid and more extensive compared to the control, such that t_{max} occurred earlier at 38min compared to 120min, C_{max} was almost doubled and the AUC increased by 58%.

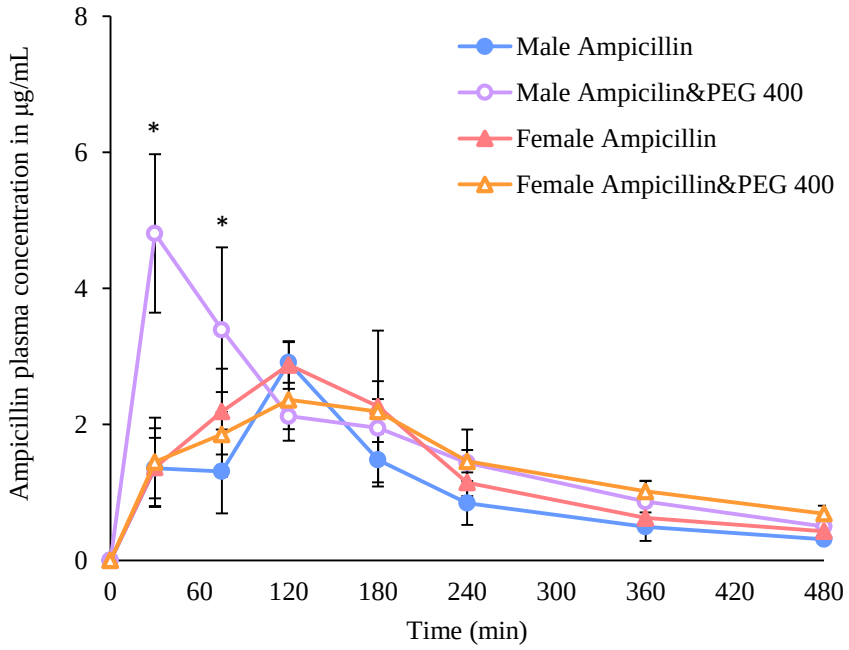


Figure 2.9 Plasma concentration-time profile of ampicillin in the absence and presence of PEG 400 over 8 hours in male and female Wistar rats (Mean ± S.D., n=6).* Value is statistically different at $p<0.05$.

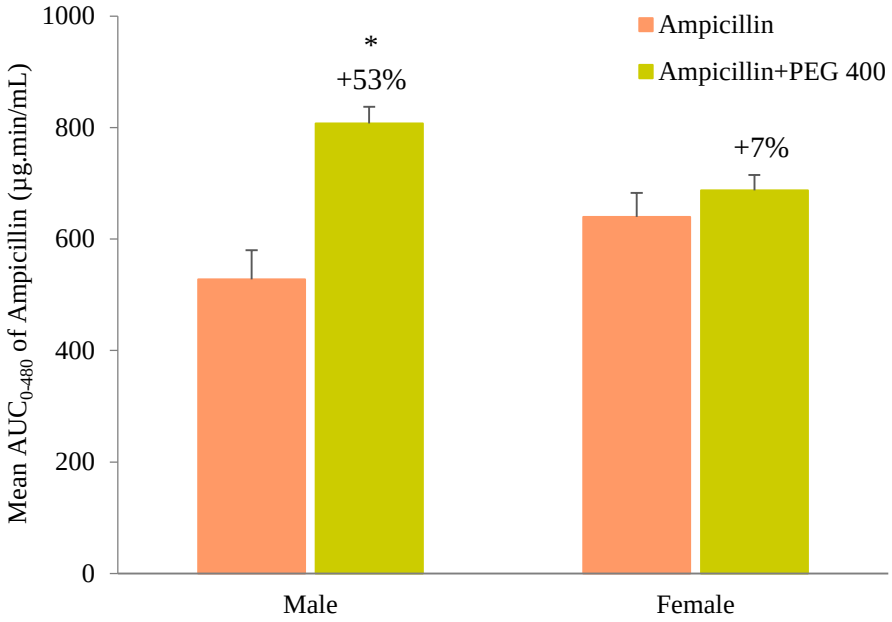


Figure 2.10 Mean $AUC_{0\text{-}480}$ of ampicillin in the presence and absence of PEG 400 in male and female Wistar rats (Mean ± S.D., n=6). *Values are statistically different between the control and PEG groups at $p<0.05$.

Table 2.10 Influence of 26mg/kg PEG 400 on the pharmacokinetic parameters of ampicillin in male and female rats (mean±S.D., n=6).

Pharmacokinetic Parameters	**Male**		**Female**	
	Control	**PEG 400**	**Control**	**PEG 400**
$AUC_{0\text{-}480}$ (*µg.min/mL*)	527.93±52.34	807.47±29.81*	639.87±42.86	687.22±27.96
AUC_{∞} (*µg.min/mL*)	577.39±74.46	939.49±57.86*	733.55±30.97	896.59±71.83
c_{max} (*µg/mL*)	2.99±0.30	5.17±0.69*	3.28±0.63	2.63±0.22
t_{max} (*min*)	108.75±22.50	37.50±18.37*	122.50±33.43	144.00±32.86
CL (*mL/min*)	0.02±0.003	0.01±0.0008	0.02±0.0007	0.01±0.003
Vd (*mL*)	4.84±1.01	3.07±0.35	3.64±0.70	5.20±2.25
$t_{1/2}$ (*min*)	156.05±43.29	160.50±26.68	147.86±28.30	209.93±33.74

* Values are statistically different between the control and PEG groups at $p<0.05$.

This sex-based influence of PEG 400 on ampicillin absorption was the same as that seen with ranitidine, where the bioavailability of the latter was increased by 49% in male rats but was unchanged in female (Afonso-Pereira et al., 2016). The similar increase in drug bioavailability in male rats for ranitidine and ampicillin indicate that the mechanisms responsible for the sex-specific influence of PEG 400 may be the same. The absorption of ranitidine and ampicillin are known to be controlled by the intestinal efflux transporter, P-gp (Collett et al., 1999, Siarheyeva, 2006), while PEG 400 is also known to influence the P-gp efflux transporter (Cook and Hirst, 1994). From this knowledge, we hypothesize that the sex-specific influence of PEG 400 on the absorption of ampicillin and ranitidine is mediated by the influence of PEG 400 on the efflux transporter P-gp. To test this hypothesis, we investigated the sex-based influence of PEG 400 on the absorption of a drug whose absorption is not controlled by P-gp. Metformin (a non-Pgp substrate) was consequently chosen as a model drug and its absorption in the absence and presence of PEG 400 is discussed in the following section.

2.3.3.3 Metformin

The influences of PEG 400 on metformin are shown in Figures 2.11, Figure 2.12 and Table 2.11. It can be seen that the presence of PEG 400 caused no change in metformin bioavailability in either males or females ($p>0.05$). As expected, any influence of PEG 400 on P-gp had no resulting effect on the absorption of metformin, which is not a substrate for P-gp.

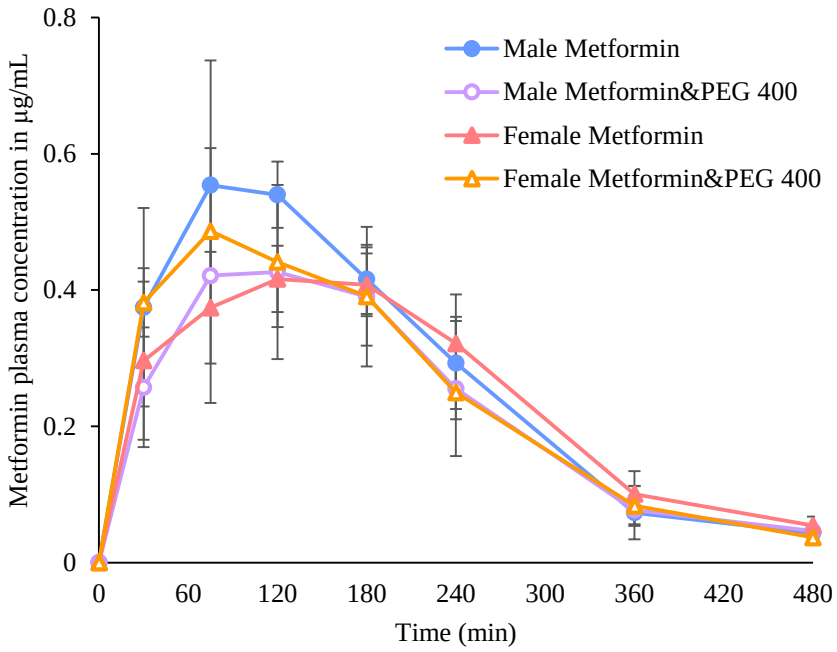


Figure 2.11 Plasma concentration-time profile of metformin in the absence and presence of PEG 400 over 8 hours in male and female Wistar rats (Mean ± S.D., n=6).* Value is statistically different at $p<0.05$.

Briefly, the metformin $AUC_{0\text{-}480}$ for the male and female rats was 31.2±5.22µg.min/mL and 39.7±11.2µg.min/mL, respectively. The bioavailability of metformin was decreased by 5.38% in the presence of PEG 400 in the male group. However, the metformin $AUC_{0\text{-}480}$ in the female group noted a different trend compared to the male partners. It increased to 47.5±8.1µg.min/mL (19.7%) with no statistical significance obtained in both male and female PEG 400 groups.

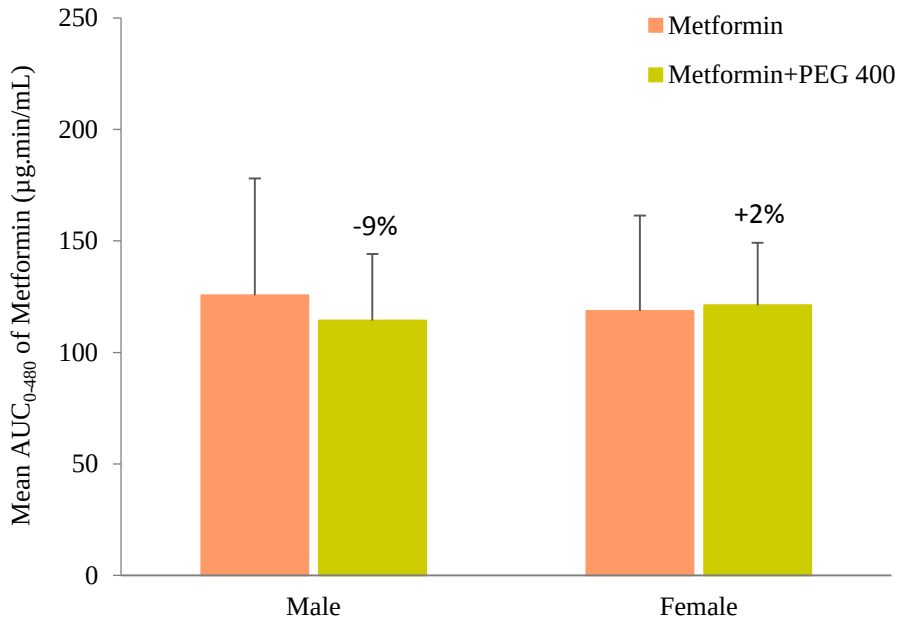


Figure 2.12 Mean AUC_{0-480} of metformin in the presence and absence of PEG 400 in male and female Wistar rats (Mean ± S.D., n=6). *Values are statistically different between the control and PEG groups at $p<0.05$.

Table 2.11 Influence of 26mg/kg PEG 400 on the pharmacokinetic parameters of metformin in male and female rats (mean±S.D., n=6).

Pharmacokinetic Parameters	**Male**		**Female**	
	Control	**PEG 400**	**Control**	**PEG 400**
AUC_{0-480} (*µg.min/mL*)	125.68±12.03	114.30±19.17	118.53±11.93	121.16±12.95
AUC_{∞} (*µg.min/mL*)	130.02±12.00	117.47±20.04	128.79±13.97	125.35±14.00
c_{max} (*µg/mL*)	0.61±0.13	0.49±0.14	0.45±0.06	0.51±0.07
t_{max} (*min*)	97.50±24.65	125.00±47.12	135.00±59.25	75.00±31.82
CL (*mL/min*)	0.09±0.008	0.11±0.02	0.10±0.01	0.10±0.01
Vd (*mL*)	13.30±1.98	18.88±5.63	18.24±3.09	14.95±3.52
$t_{1/2}$ (*min*)	100.08±14.80	118.80±22.83	129.28±17.81	102.03±14.04

Our data on ampicillin and metformin perfectly support the hypothesis that the sex differences in the effect of PEG 400 on the bioavailability of ranitidine was attributed to the sex-associated interaction between PEG 400 and P-gp. However, the presence of PEG 400 did not show sex-dependent impact on the bioavailability of P-gp-mediated fexofenadine.

Moreover, the tested drugs are also substrates for other transporters in liver or kidney. Briefly, metformin was reportedly modified by PMAT which is expressed in human intestine; OCT-2 in kidney and OCT-1 in liver (Kimura et al., 2005a, Kimura et al., 2005b, Terada et al., 2006). Ampicillin was also reported to upregulate xCT and GLT-1 isoforms expression (Alasmari et al., 2015). Therefore, our hypothesis is needed to be further confirmed, and proceeded to Phase II.

Phase II

2.4 MATERIALS AND METHODS

2.4.1 Materials

Ampicillin sodium and cyclosporine A was obtained from VWR International (Lutterworth, UK) and Cambridge Bioscience (Cambridge, UK) respectively. Ranitidine hydrochloride and polyethylene glycol 400 were supplied by Sigma-Aldrich (Dorset, UK). HPLC-grade reagents such as acetonitrile, methanol and glacial acetic acid were purchased from Fisher Scientific (Loughborough, UK). Analytical grade reagents such as ammomium acetate and sodium dihydrogen phosphate were procured from VWR International (Lutterworth, UK).

2.4.2 Animals

All the animal work was approved by the UCL School of Pharmacy's ethical review committee and was conducted in accordance with the home office standards under the Animals (Scientific Procedures) Act, 1986. Healthy male and female, 8 to 13 weeks old Wistar rats (Harlan UK Ltd, Oxfordshire, UK) weighing 150-250 g were used. The rats were housed at controlled temperatures (25℃) and humidity (50-60%) with a constant light-dark cycle of 12h, provided with food and water, and were acclimatized for 7 days before being studied.

2.4.3 Time-dependent inhibition study in ranitidine

The day before the experiment, all the rats were fasted overnight and individually housed in metabolic cages.

Each rat was weighed on the day of the experiment and administered orally with 50mg/kg CsA solution. 0min, 15min, 30min or 60min later, they were administered 50mg/kg ranitidine using an oral gavage syringe. Then, the rats were placed individually in a metabolic cage and were allowed to move freely until blood collections.

The appropriate period of CsA administration prior to drug treatment (which was used in the following ampicillin and metformin study) was chosen based on the CsA pre-treatment timing which could result to the greatest enhancement in ranitidine bioavailability in this section.

2.4.4 Effect of CsA on the ampicillin and metformin bioavailability

The rats were orally administered an appropriate volume of cyclosporine A suspension for a dose of 50mg/kg. 15 minutes later, a solution of ampicillin, metformin or ranitidine at a dose of 50mg/kg in the absence or presence of PEG 400 (at 26mg/kg) was administered via oral gavage. After dosing, approximately 250μL-300μL of blood was collected from the tail vein of rats into anticoagulant centrifuge tubes (BD Microtainer® K2E Becton, Dickinson and Company, USA) at 0.5, 1.25, 2, 3, 4 and 6h. At 8h post-administration, the rats were sacrificed with a CO2 euthanasia chamber and about 1mL of blood was taken via cardiac puncture immediately.

15 minutes before drug administration was chosen as the appropriate time to give the P-gp inhibitor, according to the study on the influence of timing using ranitidine as the drug in Section 2.4.3.

2.4.5 Sample preparation

Blood samples were centrifuged at 10,000rpm for 10min, and the supernatants (plasma samples) were collected into 1.5mL Eppendorf tubes.

For ranitidine, the samples were prepared using a reported method (Afonso-Pereira et al., 2016). 50µL of the supernatant was placed into a 1.5mL Eppendorf tube, and the same volume of acetonitrile was added to precipitate the plasma proteins. After 1 min of vortex-mixing, 100µL HPLC grade water was added to the mixture, which was vortex-mixed again for 30s, and centrifuged at 4ºC for 10 min at 10000rpm. The supernatant was collected and 40µL aqueous was analyzed by HPLC. For the ampicillin and metformin samples, the methods were described in phase I.

2.4.6 Chromatographic analysis

The amount of drug in the withdrawn samples was quantified using a high performance liquid chromatography (HPLC) system (Agilent Technologies, 1260 Infinity) equipped with a pump (model G1311C), autosampler (model G1329B) and a diode-array UV detector (model G1314B).

The ranitidine sample was subjected to HPLC-UV analysis using a previously validated method (Ashiru et al., 2007). The column used was a 5µm Luna SCX (Phenomenex, UK); the mobile phase was a mixture of 20:80 (acetonitrile):(0.1M sodium acetate pH=5.0) with a flow rate of 2ml/min and 40µL of injection volume. Methods for ampicillin and metformin samples were described in phase I.

2.4.7 Pharmacokinetic analysis

Pharmacokinetic parameters, (C_{max}, t_{max}, $AUC_{0\text{-}480}$, AUC_{∞}, CL, Vd and $t_{1/2}$) were calculated by non-compartmental analyses using a free Microsoft Excel add-in, "PKSolver." (Zhang et al., 2010).

2.4.8 Statistical analysis

All results are expressed as mean ± SD (n = 6). The control and test group data were analyzed by one-way ANOVA, followed by post-hoc Tukey analysis with a 95% confidence interval using IBM SPSS Statistics 16 (SPSS Inc., Illinois, USA). Repeated measures ANOVA was conducted to assess any statistically significant differences along the absorption profiles.

2.5 RESULTS AND DISCUSSION

2.5.1 Time-dependent inhibition study in ranitidine

A greater response to the effect of CsA on the ranitidine bioavailability was observed in males compared with female ones (shown in Table 2.12 and Figure 2.13). When the male rats were pre-treated with CsA 0, 15, 30 and 60min before being given ranitidine, the ranitidine $AUC_{0\text{-}480}$ was enhanced by 84%, 113%, 103% and 88% compared with the control (which only received ranitidine). Pronounced bioavailability enhancement was obtained with the 15min earlier treatment of CsA before ranitidine administered, a 113% increase ($p<0.05$).

For the females, significant increases in mean ranitidine $AUC_{0\text{-}480}$ by CsA were also observed, where increases of 38%, 42%, 46% and 50% over the control were found in the presence of 0, 15, 30 and 60min CsA pre-treatment. Most significant increase was found in 60min pre-treatment with CsA, which was not consistent with that in males (shown in Figure 2.14).

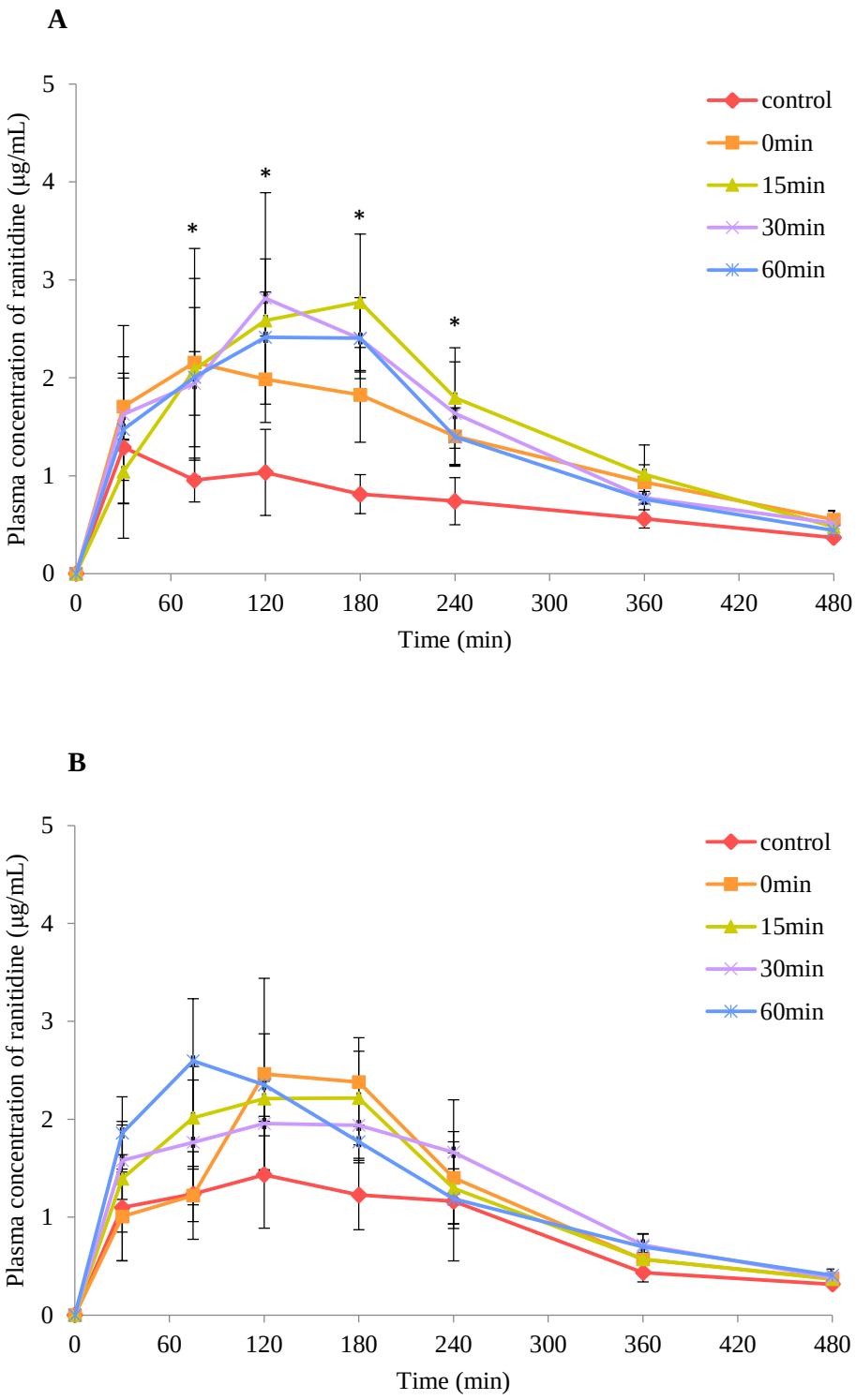


Figure 2.13 Plasma concentration-time curve of ranitidine over 8 hours in male (A) and female (B) rats that untreated (control), 0min, 15min, 30min and 60min pre-treated with CsA (Mean ± S.D., n=5). * Value is statistically different at $p<0.05$.

Table 2.12 The pharmacokinetic parameters of ranitidine in the male and female CsA pre-treated Wistar rats (Mean±S.D., n=5).

Pharmacokinetic Parameters	Control (in the absence of CsA)	CsA Administered Time before Given Ranitidine (min)			
		0[#]	15	30	60
Male					
AUC_{0-480} (*μg.min/mL*)	350.24 ± 32.82	646.15 ± 93.88 *	746.91 ± 39.45 *	711.11 ± 60.03 *	660.14 ± 33.34 *
AUC_{∞} (*μg.min/mL*)	569.72 ± 164.76	875.44 ± 175.70*	947.89 ± 70.99 *	888.52 ± 112.68 *	809.98 ± 62.42 *
c_{max} (*μg/mL*)	1.59 ± 0.75	2.57 ± 0.89 *	2.89 ± 0.58 *	3.31 ± 0.48 *	2.77 ± 0.21 *
t_{max} (*min*)	116.25 ± 90.31	126.00 ± 52.61	168.00 ± 50.20	114.00 ± 53.67	135.00 ± 45.00
CL (*mL/min*)	0.02 ± 0.005	0.01 ± 0.003	0.01 ± 0.001	0.01 ± 0.002	0.02 ± 0.001
Vd (*mL*)	11.83 ± 5.13	5.63 ± 1.29 *	5.45 ± 1.13 *	4.67 ± 0.60 *	5.02 ± 1.39 *
$t_{1/2}$ (*min*)	406.56 ± 37.35	276.02 ± 100.84*	288.27 ± 70.05 *	231.61 ± 46.91 *	228.57 ± 83.04 *
Female					
AUC_{0-480} (*μg.min/mL*)	421.26 ± 49.15	581.65 ± 66.75 *	599.27 ± 39.06 *	616.04 ± 46.17 *	631.51 ± 26.55 *
AUC_{∞} (*μg.min/mL*)	526.07 ± 64.79	723.87 ± 46.88 *	702.80 ± 60.89 *	735.66 ± 47.23 *	736.63 ± 29.89 *
c_{max} (*μg/mL*)	1.88 ± 0.27	2.93 ± 0.68 *	2.56 ± 0.33 *	2.38 ± 0.13	2.84 ± 0.48 *
t_{max} (*min*)	117.00 ± 78.23	156.00 ± 32.86	123.00 ± 37.35	138.00 ± 71.47	93.00 ± 24.65
CL (*mL/min*)	0.02 ± 0.003	0.02 ± 0.001	0.02 ± 0.002	0.02 ± 0.001	0.02 ± 0.0007
Vd (*mL*)	7.85 ± 1.21	6.52 ± 2.10	4.92 ± 0.95	5.24 ± 1.00	4.29 ± 0.58
$t_{1/2}$ (*min*)	230.60 ± 53.60	261.15 ± 84.76	193.31 ± 51.18	214.69 ± 47.71	175.56 ± 29.20

Administer the same time with ranitidine

* Values are statistically different between the control and CsA groups at $p<0.05$.

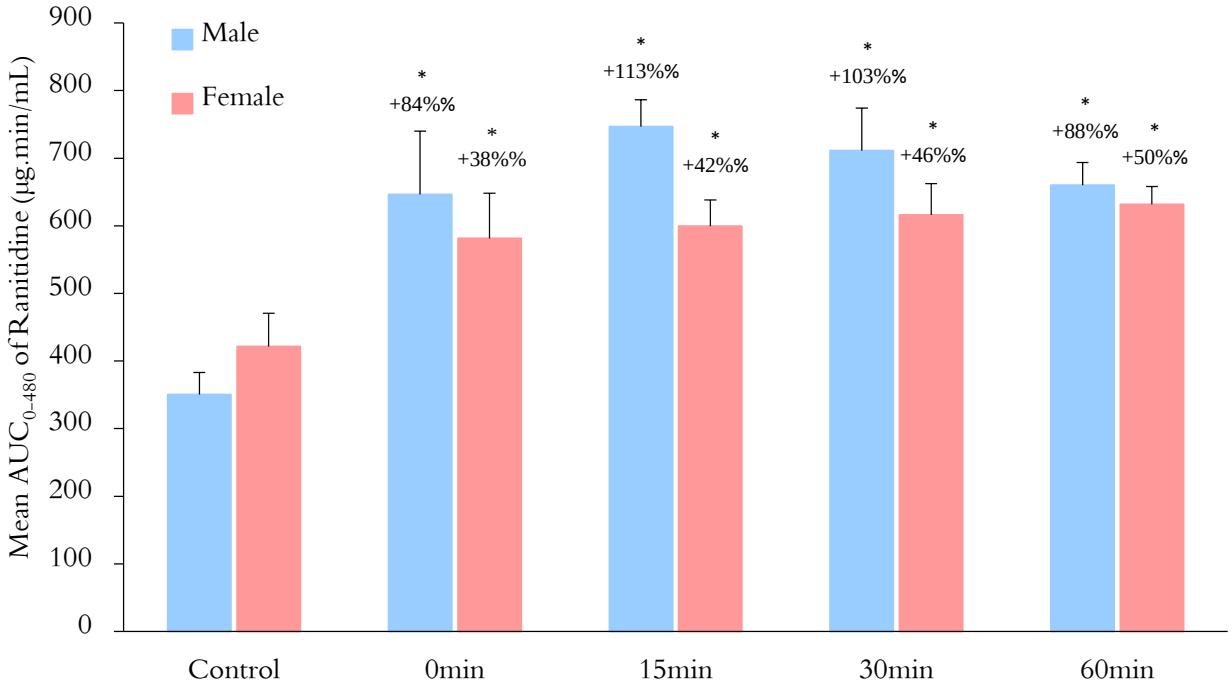


Figure 2.14 Mean AUC_{0-480} of ranitidine in the absence and presence of CsA with 0, 15, 30 and 60min pre-treatment (Mean±S.D., n=5).

* Values are statistically different between the control and CsA groups at $p<0.05$.

2.5.2 Influence of CsA on the bioavailability of ranitidine, ampicillin and metformin

We hypothesized that if the sex-related influence of PEG 400 on the bioavailability of certain drugs was a result of its influence on P-gp, blocking the latter with a P-gp inhibitor would remove the effect of PEG 400 on the bioavailability of the P-gp substrates (ampicillin and ranitidine), but would have no influence on the absorption of the non-P-gp substrate (metformin). To test this hypothesis, male and female rats were pre-treated with the P-gp blocker CsA at 15min prior to administration of ampicillin, ranitidine or metformin in the absence or presence of PEG 400.

As expected, pre-treatment with CsA did not change the bioavailability of metformin in either male or female rats ($p>0.05$) (shown in Table 2.13 and Figure 2.15). In contrast, pre-treatment with CsA increased ampicillin bioavailability in both males and females ($p<0.05$), with the percentage increase in bioavailability being higher in males than in females (132% vs 42%). Addition of PEG 400 in CsA pre-treated rats had no significant effect on the drug bioavailability in either male or female rats, compared to the CsA pre-treated rats ($p>0.05$) (Table 2.13 and Figure 2.16), i.e. once P-gp have been blocked by CsA, PEG 400 had no influence as its site of action was not available. The results are in a good agreement with our hypothesis that sex-specific influence of PEG 400 on drug bioavailability are mediated via the action of PEG 400 on the efflux transporter P-gp.

Similar results were observed for ranitidine (Table 2.13 and Figure 2.17). When the rats were pre-treated with CsA 15min before being given ranitidine, the ranitidine $AUC_{0\text{-}480}$ was increased by 113% in males and 42% in females, respectively, compared with the controls (which only received ranitidine). Meanwhile, in the CsA

pre-treated rats, no effect of PEG 400 on ranitidine bioavailability was observed in either male or female rats.

Table 2.13 Effect of CsA on the pharmacokinetic parameters of ampicillin, ranitidine and metformin in male and female Wistar rats (Mean±S.D., n=5). * Values are statistically different between the control and CsA groups at $p<0.05$.

Pharmacokinetic	**Male**		**Female**	
Parameters	**Control**	**50mg/kg CsA**	**Control**	**50mg/kg CsA**
Ampicillin				
AUC_{0-480} (*μg.min/mL*)	528±52	1209±186 *	640±43	892±89 *
AUC_{∞} (*μg.min/mL*)	577±74	1287±185 *	734±31	1003±57 *
c_{max} (*μg/mL*)	3±0.3	6±1 *	3±0.6	4±1
t_{max} (*min*)	109±23	105±56	122±33	123±37
CL (*mL/min*)	0.02±0.003	0.01±0.001	0.02±0.0007	0.013±0.0008
Vd (*mL*)	5±1	2±0.4	4±0.7	4±1
$t_{1/2}$ (*min*)	156±43	137±17	148±28	196±60
Ranitidine				
AUC_{0-480} (*μg.min/mL*)	350±33	747±39 *	421±49	599±39 *
AUC_{∞} (*μg.min/mL*)	570±165	948±71 *	526±65	703±61 *
c_{max} (*μg/mL*)	2±0.8	3±0.6 *	2±0.3	3±0.3 *
t_{max} (*min*)	116±90	168±50	117±78	123±37
CL (*mL/min*)	0.02±0.005	0.01±0.001	0.02±0.003	0.02±0.002
Vd (*mL*)	12±5	5±1 *	8±1	5±1
$t_{1/2}$ (*min*)	407±37	288±70 *	231±54	193±51
Metformin				
AUC_{0-480} (*μg.min/mL*)	126±12	127±18	119±12	106±20
AUC_{∞} (*μg.min/mL*)	130±12	136±18	129±14	147±29
c_{max} (*μg/mL*)	0.6±0.1	0.6±0.06	0.5±0.06	0.5± 0.1
t_{max} (*min*)	98±25	105±46	135±59	138±58
CL (*mL/min*)	0.09±0.008	0.09±0.01	0.1±0.01	0.09±0.01
Vd (*mL*)	13±2	19±3	18±3	16±4
$t_{1/2}$ (*min*)	100±15	142±16	129±18	150±39

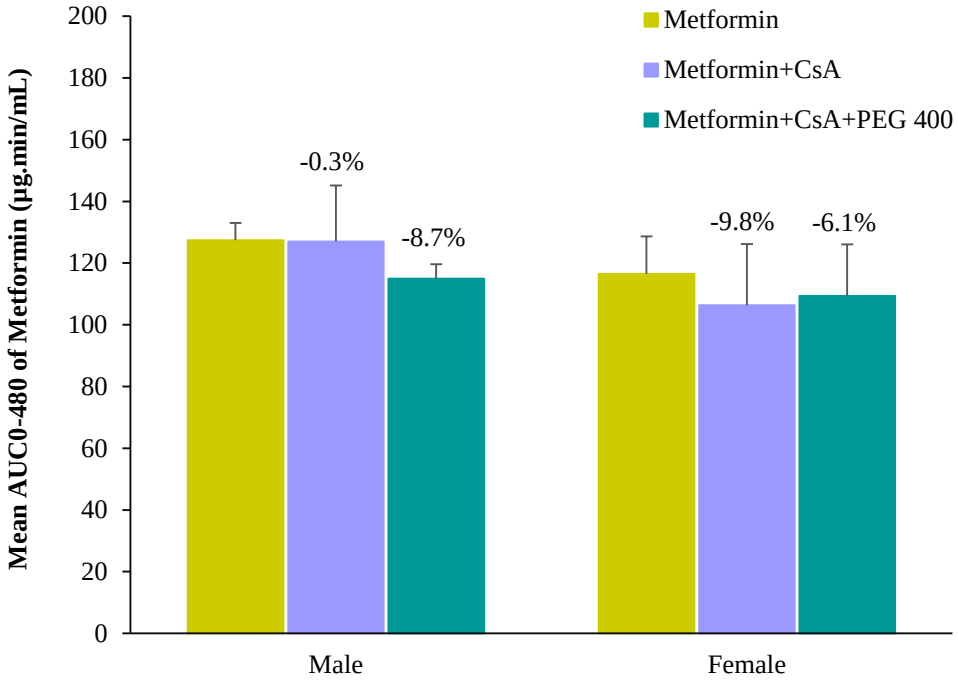


Figure 2.15 Mean $AUC_{0\text{-}480}$ of metformin in the presence or absence of PEG 400 in male and female cyclosporine A pre-treated rats (Mean ± S.D., n=6).

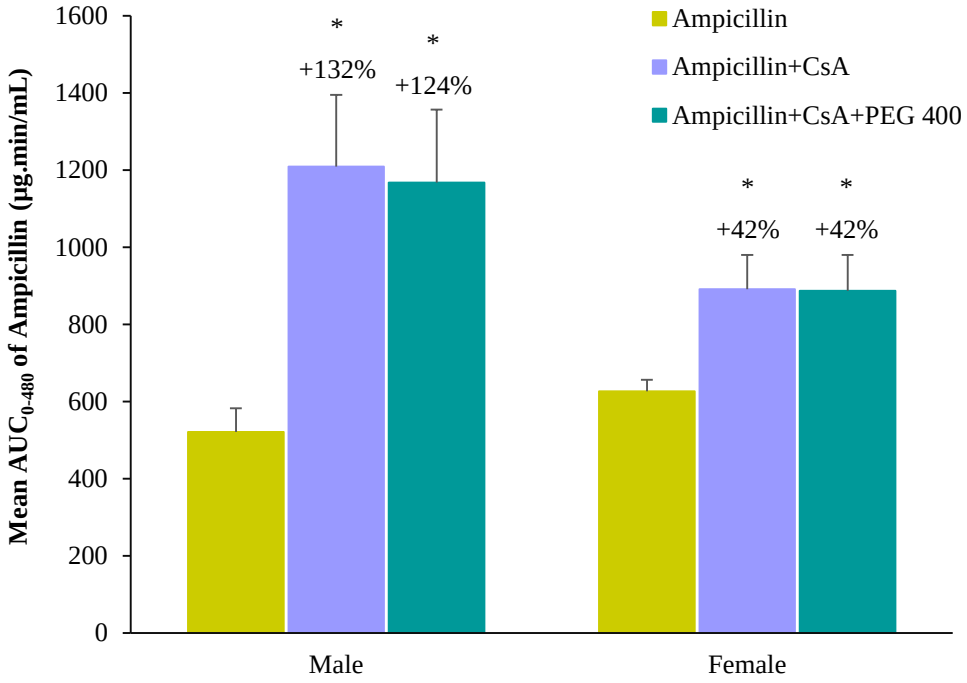


Figure 2.16 Mean $AUC_{0\text{-}480}$ of ampicillin in the presence or absence of PEG 400 in male and female pre-treated rats with cyclosporine A (Mean ± S.D., n=6). *Values are statistically different from those in the control and tested groups at $p<0.05$.

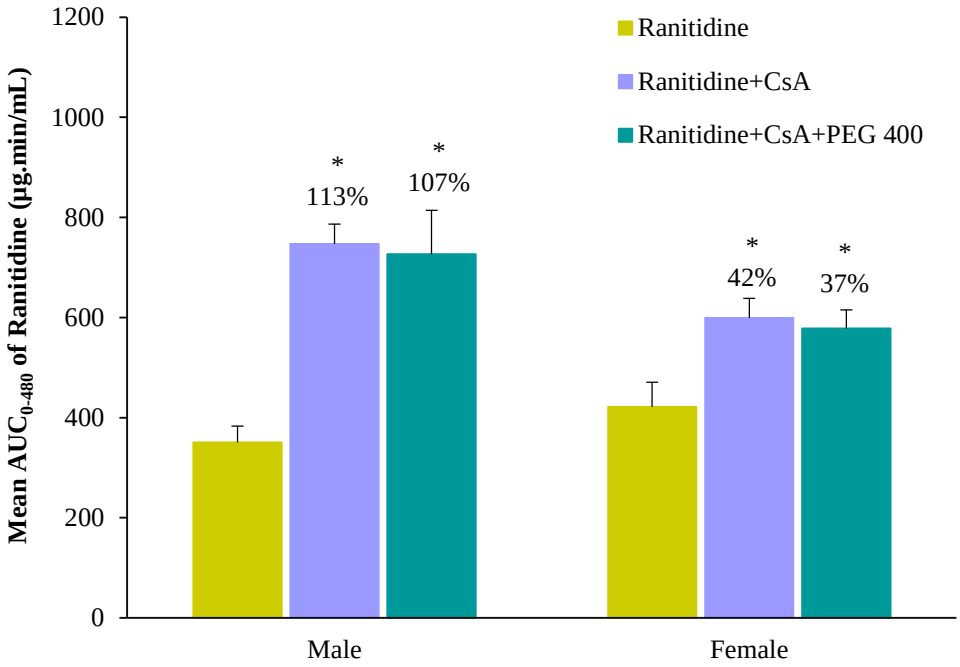


Figure 2.17 Mean AUC_{0-480} of ranitidine in the presence or absence of PEG 400 in male and female pre-treated rats with cyclosporine A (Mean ± S.D., n=6). *Values are statistically different from those in the control and tested groups at $p<0.05$.

As seen in the results, the influence of PEG 400 and CsA on the bioavailability of P-gp substrates depends on the sex of the animals, i.e. a greater response was observed in male rats compared to female ones, suggesting higher P-gp activity and/or expression in male rats than female ones.

Higher P-gp activity in males compared to females has previously been suggested in a study, where the effect of PSC833 (a P-gp inhibitor) on the intestinal transport of two P-gp-mediated drugs, ivermectin and Rho 123, was investigated using tissues from male and female rats (Ballent et al., 2012, Mariana et al., 2011). Greater ivermectin accumulation was observed in male intestines (by 141%) compared to female ones (by 14%). Similar results were observed for Rho 123, in which its efflux ratio significantly decreased from 4.33 to 1.51 in males ($p<0.05$) but not in

females. While the reasons for greater P-gp activity in males compared to females have not been established, we speculate that the differential P-gp activity is modulated by P-gp modifying-mechanisms which may themselves be influenced by the sex of organism. For example, CsA alters the P-gp activity by inhibiting both the substrate stimulated- and the basal- P-gp ATPase (Watanabe et al., 1997), while PEG influences the function of P-gp by producing mitochondrial toxicity and depleting the amount of intracellular ATP (Johnson et al., 2002). Sex-related differences in the variations in P-gp ATPase or ATP level in cells could cause sex differences in the interaction between CsA/PEG and P-gp. Further work is needed to clarify the mechanisms underlying this phenomenon.

In addition to P-gp activity, sex-related differences in P-gp expression could also explain our results. Sex difference in the expression of P-gp was first proposed in liver (Schuetz et al., 1995b) and later reported in the intestine (Gerrard et al., 2004), where men were reported to have higher hepatic and enterocyte P-gp content than women. However, no sex-based difference in P-gp expression have been proposed in the upper duodenum (Paine et al., 2005b). Meanwhile, no sex-related differences in the intestinal P-gp protein content and mRNA expression were found (MacLean et al., 2008). The fact that rats were fed in this study (while fasted rats were used in our in vivo studies) shows that the possibility of sex-based differences arising in the intestines of fasted rats cannot be excluded, given that the fed/fasted states have been shown an impact on P-gp and other membrane transporters (Deferme and Augustijns, 2003, Deferme et al., 2003, Furumiya and Mizutani, 2008).

P-gp activity has been found to be largely similar in rats and humans (Li et al., 2015, Li et al., 2017). Based on this, the observed influence of PEG 400 (in this paper) on the bioavailability of P-gp substrates has implications for drug usage in humans. Indeed, we have already shown differential effects of PEG 400 on the oral bioavailability of ranitidine in men and women (Ashiru et al., 2008). It suggested

that the bioavailability of a drug which is a P-gp substrate and which is formulated with PEG 400 as an excipient will be higher in men compared to women, with the possibility that the therapeutic level will not be reached in women.

2.6 CONCLUSION

The work reported in this chapter further enhances our understanding of the influence of the supposedly inactive excipient PEG 400 on drug bioavailability, specifically its different action depending on the sex of the organism. We confirmed that PEG 400 had a greater drug bioavailability-enhancing influence in males compared to females, such as ranitidine and ampicillin. Importantly, the sex-related influence of PEG 400 occurred only for drugs whose absorption is controlled by the efflux transporter P-gp. Thus, blocking of P-gp by cyclosporine A (a P-gp inhibitor) eliminated the effect of PEG 400 on the bioavailability of ampicillin and ranitidine. Cyclosporine A blocking of P-gp had its own sex-related effect on drug bioavailability. This differential sex-based influence on drug bioavailability could be due to greater P-gp activity and/or expression in males compared to females, as suggested by some existing literature. Given that many compounds are P-gp substrates and/or modulate P-gp activity, increasing attention to this topic is needed for an optimal usage of excipients.

CHAPTER 3: Predictive Techniques: Is *in vitro* data sufficient to correlate PEG 400 *in vivo* effects on drug absorption?

3.1 INTRODUCTION

The hypothesis in chapter 2 (the reason behind PEG's sex-specific impact on the ranitidine bioavailability was attributed to a greater P-gp function in males compared to females) was suggested according to the previous *in vivo* studies. In light of countless physiological factors involved in oral drug absorption *in vivo* (namely, GI transmit time as well as the activity and composition of intestinal bacteria), an *in vitro* study was required in order to further explore the reason behind the sex-related effect of PEG 400 on ranitidine absorption. Similarly, such an *in vitro* method could also be used to investigate the sex-dependent influence of other excipients on drug transport in animal models or even humans. Consequently, the aim of the study in this chapter was to explore the impact of PEG 400 on the permeation of ranitidine in the absence and presence of P-gp inhibitor and cyclosporine A (CsA) in four intestinal divisions of male and female rats with the Ussing chamber system, which has been widely used in evaluating intestinal drug permeation.

Current approaches characterizing drug absorption via the intestine have been conduction on a variety of models: *in vitro* (tissue- or cultured cell-based), *in situ* and *in vivo* intestinal perfusion, and *in silico* methods (Hillgren et al., 1995, Le Ferrec et al., 2001, Reis et al., 2013). For many reasons, although particularly due to financial and ethical considerations, *in vitro* approaches have been pursued in this area for decades due to the good agreement between intestinal permeability results in specific animals and human subjects (Luo et al., 2013, Antunes et al., 2013).

The purpose of any *in vitro* model is to perform various studies under well-controlled and easily accessed conditions without using living animals (Roeselers et al., 2013). Moreover, for mechanistic studies aiming to elucidate cellular processes, a simple cell-line model may be accurate. However, feedback mechanisms are lost in most *in vitro* cell culture models. Additionally, cell culture models cannot reflect sex-related characters, which is a necessity in our studies. Therefore, if extrapolating *in vitro* data to the *in vivo* situation is required, the model

must reflect the complexity of the questions being asked. In this context, functional organs or tissue *ex vivo* show extensive interaction and crosstalk with different cell lines.

In *in vitro* tissue-based models, also called *ex vivo* models, experiments include procedures with living functional tissues or organs isolated from an organism and cultivated outside the organism in an artificial environment under highly controlled conditions (Agu et al., 2008).

3.1.1 Ussing Camber

The Ussing chamber technique was first developed by Ussing and Zerahn (1951) to study transepithelial ion transport across frog skin, then later adapted by Grass and Sweetana (1988) (Sweetana-Grass diffusion chamber) to evaluate the intestinal permeability of drugs. So far, several modifications of the classical Ussing chamber were made to improve the performance of this model. Currently, several variants of the Ussing chamber are available for permeability studies across epithelial tissues. They can vary in size, volume and area of exposed tissue (Clarke, 2009).

In this model, a section of intestinal tissue is excised, cut in small segments of an appropriate size and opened to form a flat epithelial sheet that is placed between the two halves of the chamber. The chambers are filled with a physiological buffer, continuously gassed with O_2/CO_2 (95%/5%) and warmed up to 37 °C (Antunes et al., 2013). The O_2/CO_2 mixture not only ensures adequate oxygenation of the tissue but also provide a layer that jeopardizes the permeability of poorly soluble compounds (Buckley et al., 2012). A schematic representation of the classical Ussing chamber is presented in Figure 3.1.

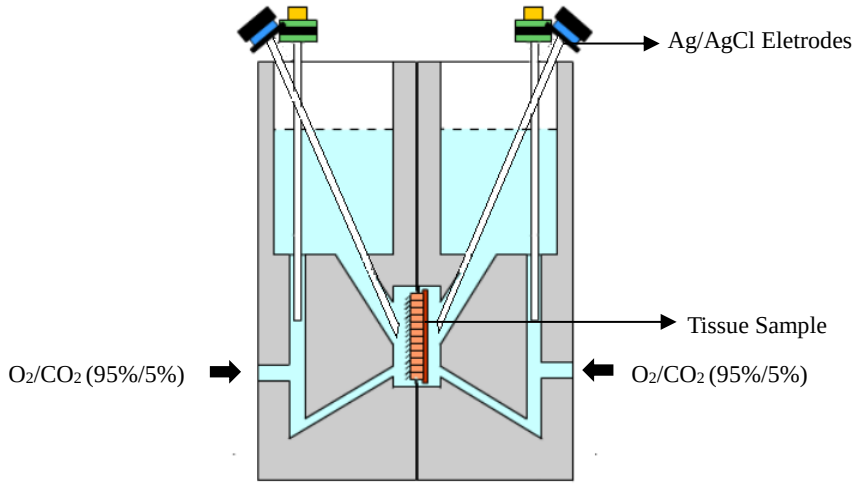


Figure 3.1 A presentation of the Ussing chamber system used for the permeation study in this chapter.

The EVOMX voltohmmeter and Ag/AgCl electrodes were used to measure the transepithelial electrical resistance (TEER) of the tissue samples. TEER monitors the presence of functional tight junction, which are responsible for the barrier function and which limit paracellular permeation of water and solutes. The resistance is measured by applying a voltage and measuring the change in current across the tissue by Ohm's law. Any duodenum, jejunum, ileum and colon segments that presented a value of TEER lower than $20\Omega \bullet cm^2$, $40\Omega \bullet cm^2$, $50\Omega \bullet cm^2$ and $70\Omega \bullet cm^2$ respectively, at the beginning of experiment was regarded poorly viable and excluded immediately (Polentarutti et al., 1999). Whenever TEER values decreased more than 15% from the value measured at the end of equilibration period, the tissue was considered not viable.

Ussing chamber experiments including tissue preparation, the choice of an adequate incubation buffer, and the motorization of tissue viability and integrity. All these factors could influence the permeability of drugs.

3.1.2 Everted Intestinal Sac

The everted gut technique was another popular *ex vivo* method, first introduced by Wilson and Wiseman (1954) to study absorption of compounds along the intestine of rat and golden hamster (Balimane et al., 2000, van de Kerkhof et al., 2007). Currently, this technique can be used as *in vitro* tool to study the mechanisms and kinetics of drug absorption. It has also been extensively explored to carry out pharmacokinetic investigations such as drug absorption, drug metabolism, multidrug resistance, drug interactions and the impact of efflux transport modulators on the absorption of drugs (Alam et al., 2013). In the everted gut model, a section of the intestine (about 2-4cm) is quickly removed from the anesthetized animal, flushed with buffer or a saline solution and everted over a rod or tube (Bohets et al., 2001, Volpe, 2010). The serosa becomes the inside of the sac and the mucosa faces the outer buffer solution (Lennernas et al., 1997). Each end of the sac is tied and filled with oxygenated buffer and placed in a container with the test drug; accumulation in the inner compartment is measured after a specified time period. The everted intestinal sac system is observed in Figure 3.2.

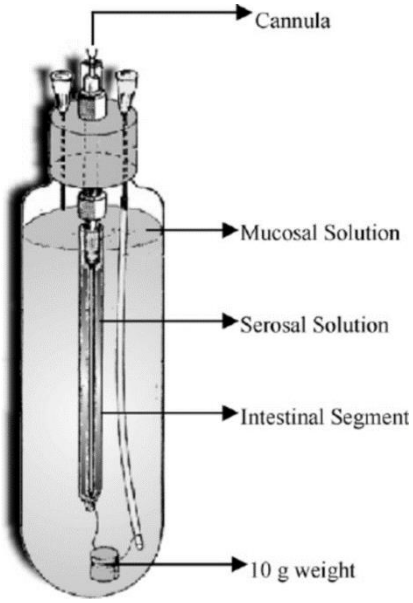


Figure 3.2 Design of the device used for the everted gut sac intestinal permeation assay, (Quevedo and Brinon, 2009).

3.1.3 Comparison

The various experimental protocols for predicting the fraction absorbed in humans or rats from permeability coefficients have their own advantages and limitations (shown in Table 3.1). Comparing with other *ex vivo* methodologies, the Ussing chamber system lends itself to fast and reproducible intestinal absorption studies, which is very useful for interspecies comparison and position specific transport, suitable *in vitro* methods in our study (Acra and Ghishan, 1991, Fortuna et al., 2012).

Table 3.1 Advantages and limitations of various *in vitro* models.

Techniques	Advantages	Limitations
Everted Intestinal Sac/Rings (Rats)	• All cell types and the mucous layer are present. • A relatively fast and inexpensive technique. • Can be used for mechanism of absorption or formulation studies.	• Not a perfused model. • The drug must cross the whole intestinal wall. • It is an animal model.
Ussing Camber (Human and Rats)	• Drug absorption and passage at specific intestinal sites (human, rat) are possible. • The test drug can be added on either the apical or the basolateral side. • Metabolism studies are possible. • A human and animal model.	• Measurement techniques must be sensitive, since the drugs are diluted in the diffusion chambers. • Cell viability is limited. • Availability of human tissue is limited. • Not used for screening.
Intestinal Perfusion (Human and Rats)	• It is a reference model. • All physiological factors that influence passage are present. • Allows studies in humans. • Good correlation with pharmacokinetic studies.	• A difficult technique with local anaesthesia at the time of catheter introduction. • Not used either in development or routinely.
In situ Techniques (Rats)	• Integrates passage and metabolism aspects. • All physiological factors that influence passage are present. • Studies of absorption in particular sites of the intestine are possible. • Studies of direct effects of the drug on intestinal absorption are possible.	• Used in development, but not routinely. • The increase of luminal hydrostatic pressure during the experiment can influence intestinal permeability. • It is an animal model.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Ranitidine hydrochloride and polyethylene glycol 400 (PEG 400) were purchased from Sigma-Aldrich (Poole, UK). Cyclosporine A (CsA) was purchased from Cambridge Bioscience (Cambridge, UK). HPLC-grade reagents such as acetonitrile and glacial acetic acid were supplied by Fisher Scientific (Loughborough, UK). Krebs-Bicarbonate Ringer's solution (KBR) was prepared with 10mM D-glucose, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 115mM NaCl, 25mM $NaHCO_3$, 0.4Mm KH_2PO_4, 2.4mM K_2HPO_4, and pH was adjusted to 7.4 with NaOH/HCl (Clarke, 2009).

3.2.2 Animals

All the animal work was approved by the UCL School of Pharmacy's ethical review committee and was conducted in accordance with the home office standards under the Animals (Scientific Procedures) Act, 1986. Healthy male and female, 8-13 week old Wistar rats (Harlan UK Ltd, Oxfordshire, UK) weighing 150-25g were used for excised rat intestine. The rats were housed at controlled temperatures (25℃) and humidity (50-60%) with a constant light-dark cycle of 12h, provided with food and water, and were acclimatized for 7 days before being studied. One day before the experiments, the rats were fasted overnight and housed individually in the metabolic cages.

3.2.3 Compound stability in an Ussing chamber system

Ranitidine solution containing 3mM drug in the absence and/or presence of PEG 400 were prepared in KBR solution. CsA was suspended in KBR buffer at 8 μM.

The stability of ranitidine and CsA in KBR solution was assessed by incubating the drug in KBR buffer at 37℃ for 180 mins, following quantifying the drugs with HPLC methods. The dose of sample withdrawn at 180 mins was compared with the drug concentration obtained at the beginning of the assay.

3.2.4 Mucosal tissue preparation

On the day of experiment, rats were sacrificed in a CO_2 euthanasia chamber and their intestines were rapidly removed. The intestine was cut into 4 segments: duodenum, jejunum, ileum and colon, which were added to beakers containing ice-cold KBR solution. The intestinal segments were allowed to rest for approximately 20 mins after lowering the tissue temperature to minimize tissue damage during preparation. Approximately 2-3cm long pieces from the mid part of the duodenum, proximal part of the jejunum, the distal to mid part of ileum and ascending colon were opened along their mesenteric border. The tissues were washed gently with KBR solution to remove the intestinal contents. To obtain the mucosal tissue, sections were placed on an ice-cold glass plate and the serosa was gently squeezed out.

3.2.5 Ussing chamber set-up

Once the mucosal tissues were well prepared, they were mounted in the vertical Ussing Chamber (Harvard Apparatus Inc., Holliston, MA, U.S.A) as flat sheets on a 0.28 cm^2 segment holder with needles to stabilize it. The mucosal surface of the tissue was facing the apical chamber, and the endothelial surface of the tissue was facing the basolateral chamber. 5 mL blank KBR solution was added to both apical and basolateral chambers, gassed with an O_2/CO_2 (95%/5%) gas mixture. The

chambers were tightly screwed with high spring-tension retaining rings and the entire assembly was kept at 37℃ with a circulating water bath for an equilibrium period.

To evaluate tissue integrity during experiments, tissue transepithelial electrical resistance (TEER) was measured using an EVOMX meter (World Precision Instruments Inc., WPI, Hertfordshire, United Kingdom) every 30min during the experiment. Any duodenum, jejunum, ileum and colon segments that presented a value of TEER lower than 20Ω•cm^2, 40Ω•cm^2, 50Ω•cm^2 and 70Ω•cm^2, respectively, at the beginning of experiment was regarded as poorly viable and excluded immediately. Whenever TEER values decreased more than 15% from the value measured at the end of equilibration period, the tissue was considered not viable.

3.2.6 Bidirectional transport

After an equilibrium period of 20-30 mins, the experiment was started by replacing the blank KBR solution with pre-warmed 3mM ranitidine solution in the donor compartment. For the absorptive transport (mucosa to serosa, M-S), the donor was the apical chamber, while for the excretive transport (serosa to mucosa, S-M), the donor was the basolateral chamber. The permeation experiment lasted 3h, and 100μL of receiver solution was withdrawn every 30min in order to measure the drug amount. An equal volume of heated blank KBR solution was added immediately after each sample was withdrawn.

3.2.6.1 Effect of PEG 400 on ranitidine permeability in the absence of CsA

This study is processed with ranitidine in the absence or presence of 0.25%, 0.5%, 1%, 1.5%, 2% or 3% PEG 400 in the apical chamber. The dose of PEG 400 that caused a pronounced ranitidine permeability enhancement was used to assay its effect on the secretive transport of ranitidine, and also to assess the influence of PEG 400 on ranitidine intestinal transport in the presence of P-gp inhibitor, CsA.

3.2.6.2 The effect of PEG 400 on ranitidine permeability in the presence of CsA

The effect of a validated P-gp inhibitor (CsA) on the bidirectional transport of ranitidine was investigated. CsA was added to the apical chamber at a final concentration of 8μM. 15min later, ranitidine was added into the apical camber (absorptive) or basolateral chamber (secretive) in the absence and/or presence of PEG 400.

15min pre-incubation with CsA before addition of drug solution was chosen as the appropriate time to give the P-gp inhibitor following a study on the influence of timing. In this study, P-gp inhibition was conducted by incubation intestinal tissue with CsA at the same time or 15min, 30min or 60min before adding 3mM ranitidine. The results showed the greatest inhibitory influence of CsA on ranitidine permeatability, when intestinal tissue was 15min pre-incubation with CsA prior to the addition of ranitidine in donor chamber.

3.2.7 Chromatographic analysis

The amount of drug in the withdrawn samples was quantified using a high performance liquid chromatography (HPLC) system (Agilent Technologies, 1260 Infinity) equipped with a pump (model G1311C), autosampler (model G1329B) and a diode-array UV detector (model G1314B).

For the analysis of ranitidine, a 5μm Luna SCX column (Phenomenex, UK) was used for sample separation using a mixture of 20:80 (acetonitrile):(0.1M sodium acetate pH=5.0) as mobile phase for elution, with a flow rate of 2mL/min (Ashiru et al., 2008, Xu et al., 2013).

In the case of CsA, separation was achieved on a 3μm Luna C8(2) column (Phenomenex, UK). The elution was isocratic at 1mL/min with a mobile phase of acetonitrile–water–phosphoric acid (750:250:1,v/v/v).

3.2.8 Calculation

The apparent permeability coefficient (P_{app}) of each experiment, in cm/s, was calculated using the equation followed:

$$Papp = \frac{Q}{C \cdot A \cdot t}$$

where Q (μmol) is the total amount of drug that permeated to the receiver compartment throughout the incubation time, C (μmol/mL) is the initial drug concentration in the donor side, A (cm^2) is the diffusion area of the Ussing Chamber, and t (s) is the time of experiment.

To study ranitidine efflux, an efflux ratio is calculated from the mean serosal-to-mucosal (S-M) P_{app} and mucosal-to-serosal (M-S) P_{app} data, as followed:

$$\text{Efflux Ratio} = \frac{Papp\ (S-M)}{Papp\ (M-S)}$$

3.2.9 Statistical analysis

The experiments were performed at least six times and data were expressed as Mean ± standard deviation (S.D.). Significant differences between groups were analysed by one-way ANOVA and three-way ANOVA using IBM SPSS Statistics 19 (SPSS Inc., Illinois, USA). A minimum p value of 0.05 was used as a significance level for the tests.

3.3 RESULTS AND DISCUSSION

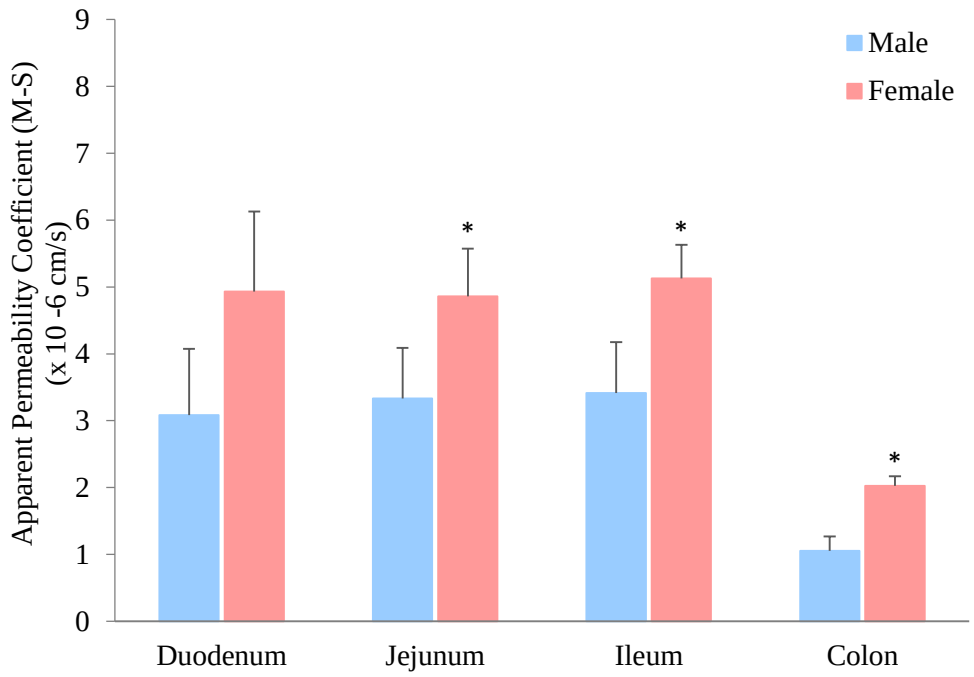
3.3.1 Influence of animal's sex on the transport of ranitidine

The absorptive and secretive permeability of ranitidine in different intestinal segments of male and female rats, presented as the apparent permeability coefficient (Papp), are shown in Figure 3.3.

Sex differences were observed for both absorption and secretion of ranitidine. Ranitidine intestinal absorption (Papp mucosa-serosa, M-S) for jejunum, ileum and colon was higher in female rats compared to male rats, while its secretion (Papp serosa-mucosa, S-M) was larger in males ($p<0.05$). Contrary to the other intestinal segments, no differences was observed between male and female duodenum.

Greater ranitidine absorption but lower secretion in females (Figure 1) would be expected to lead to higher bioavailability of ranitidine in females compared to males in vivo. Surprisingly, this was not the case, which reported no sex-based differences in the ranitidine bioavailability in rats. We speculated the contrast between in vitro and the in vivo data (Afonso-Pereira et al., 2016) is due to higher ranitidine metabolism in females compared to males. Ranitidine is metabolised by CYP3A (Penston and Wormsley, 1986), and women reportedly have higher CYP3A metabolism than men (Wolbold et al., 2003).

A



B

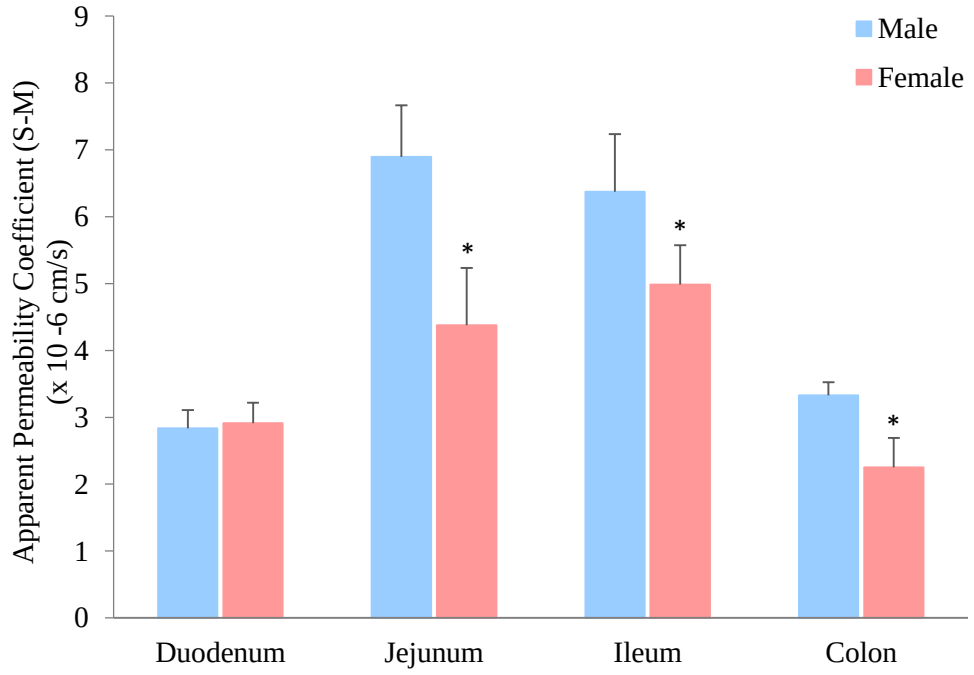


Figure 3.3 Absorptive (A) and secretive (B) transport of ranitidine across the intestine of male and female Wistar rats (Mean ± S.D., n=6). * Values are statistically different between the male and female groups at $p<0.05$.

3.3.2 Influence of intestinal region on the transport of ranitidine

Figure 3.4 and Figure 3.5 represent the time course of transport of ranitidine in different intestinal segments of male and female rats. It can be seen that the absorptive and secretive transport of ranitidine in small intestine (duodenum, jejunum and ileum) were higher than in colon ($p<0.05$). When it comes to three divisions in small intestine, the absorption of ranitidine did not exhibit a regional difference (it was similar in the duodenum, jejunum and ileum). In contrast, ranitidine secretion was found to differ between intestinal segments, which was greatest in the jejunum and ileum, and lowest in the duodenum.

Our data about the absorptive transport of ranitidine in the intestine of male and female rats supported the previous study, where ranitidine was reported to be primarily absorbed in the ileum as compared to the duodenum and jejunum (Mummaneni and Dressman, 1994).

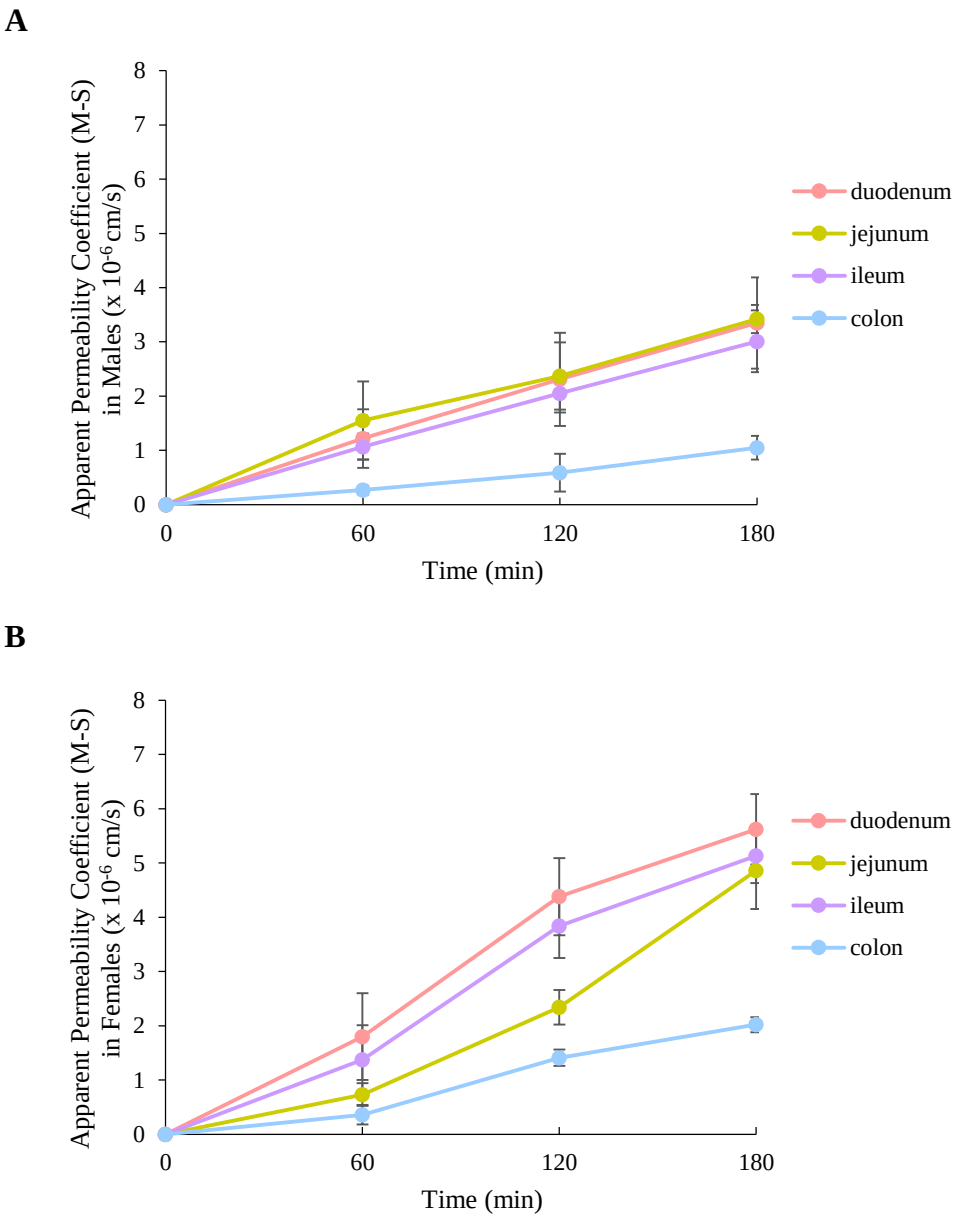


Figure 3.4 Time course of the apparent permeability coefficients considering absorption (mucosal-to-serosal, M-S) of ranitidine in the male (A) and female (B) Wistar rats (Mean ± S.D., n=6).

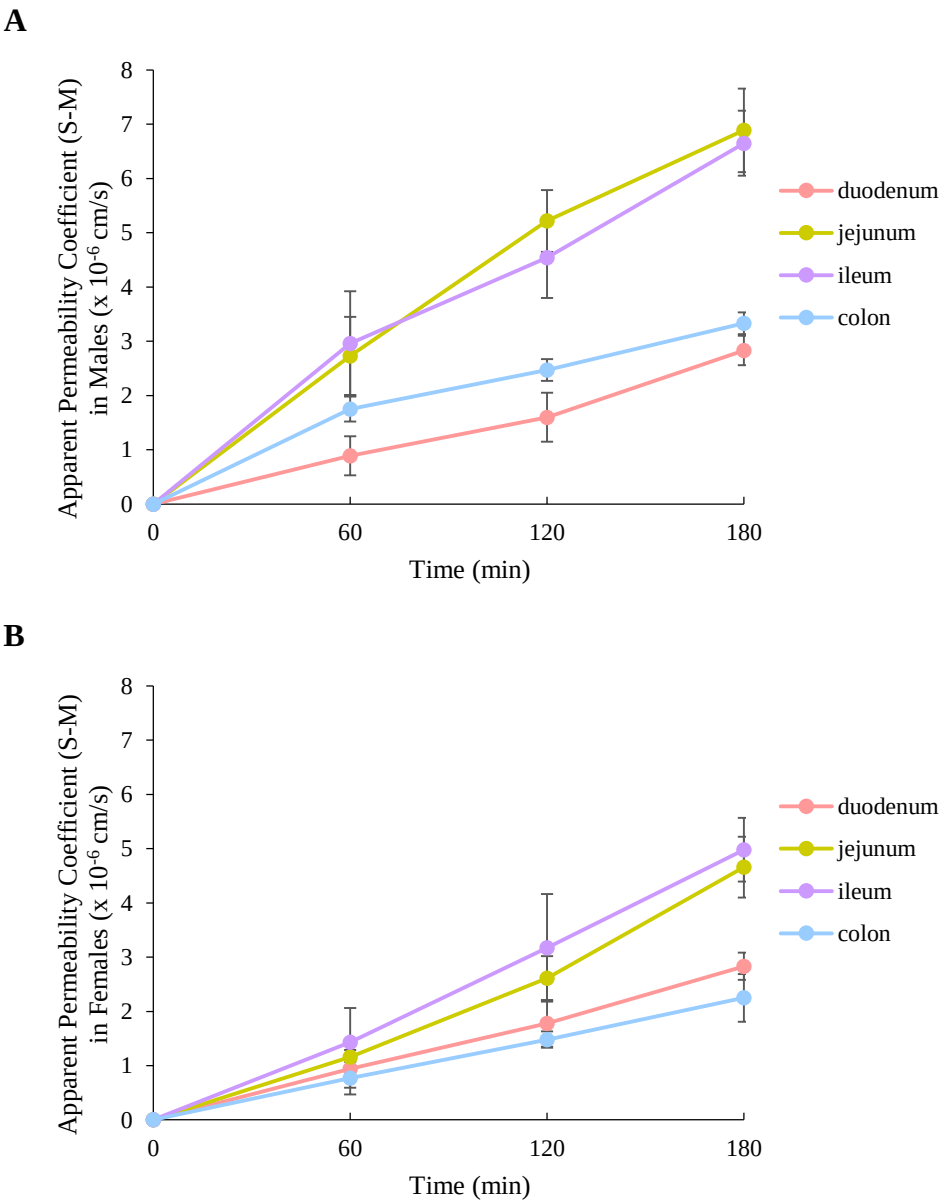


Figure 3.5 Time course of the apparent permeability coefficients considering secretion (serosal-to-mucosal, S-M) of ranitidine in the male (A) and female (B) Wistar rats (Mean ± S.D., n=6).

3.3.3 Dose-dependent influence of PEG 400 on ranitidine absorption

The presence of PEG 400 increased the intestinal absorption of ranitidine in males but not in females, which show a sex-specific manner. The effect of this 'inert' excipient PEG 400 in male rats was also dose-dependent and region-related. As shown in Figure 3.7 for the jejunum and Figure 3.6 and Figure 3.8 for the duodenum and ileum, increasing the dose of PEG 400 led to increasing and then decreasing ranitidine absorption in male rats, such that a bell-shaped effect (in relation to PEG concentration) was observed. However, a rising curve was found for male colon, as shown in Figure 3.9.

High doses of PEG 400 (>1g) reportedly reduced transit time in the small intestinal and the oral absorption of ranitidine, while a low dose of PEG 400 could increase ranitidine bioavailability in males (Schulze et al., 2003). However, our results demonstrated that the reduction of oral absorption of ranitidine with a high dose of PEG 400 may not only be due to the longer transit time in the small intestine, but also due to a reduction in the absorptive permeability of ranitidine. Its modulation of the intestinal permeability of ranitidine may be via the alteration of membrane fluidity and/or inhibition of some membrane uptake transporters. For example, it was reported that the commonly used excipients mannitol and sorbitol reduce the intestinal uptake of ranitidine and cimetidine due to high pressure on the membrane (Adkin et al., 1995a, Adkin et al., 1995b, Chen et al., 2007). Further, the inhibition of OATPs by PEG 400 in the absorption process of estrone-3-sulfate and taurocholate from the gut lumen proposed that PEG 400 might be a selective modulator of other intestinal uptake transporters (Engel et al., 2012). Likewise, a low dose of PEG 400 (such as 0.5%) resulted in the enhanced transport of ranitidine in male rats, which was in strong agreement with the human data (Ashiru et al., 2008). Therefore, the increase in the bioavailability of ranitidine with a low dose of PEG 400 in humans may potentially be because of an enhanced intestinal permeation.

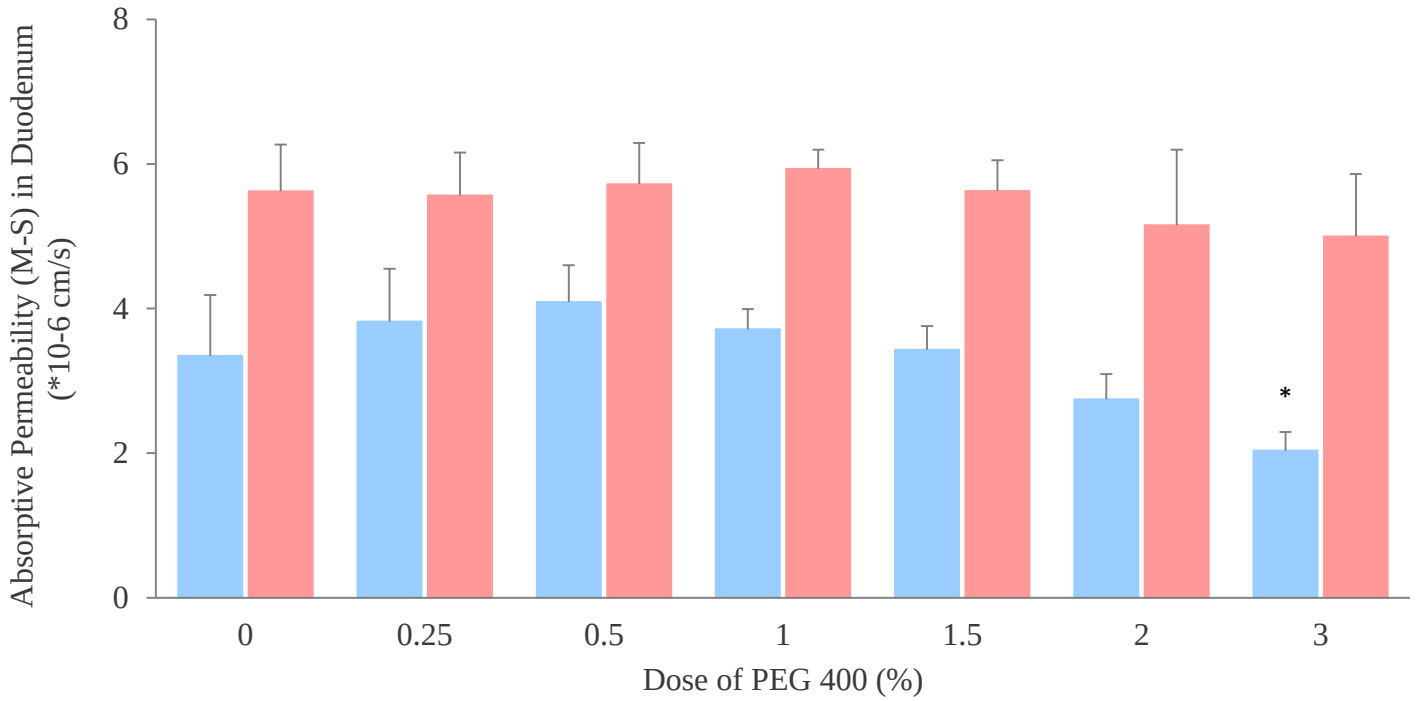


Figure 3.6 Absorptive transport of ranitidine across the duodenum of male (blue) and female (pink) Wistar rats (Mean ± S.D., n=6) in the presence of PEG 400. * Values are statistically different between the control and tested groups at $p<0.05$.

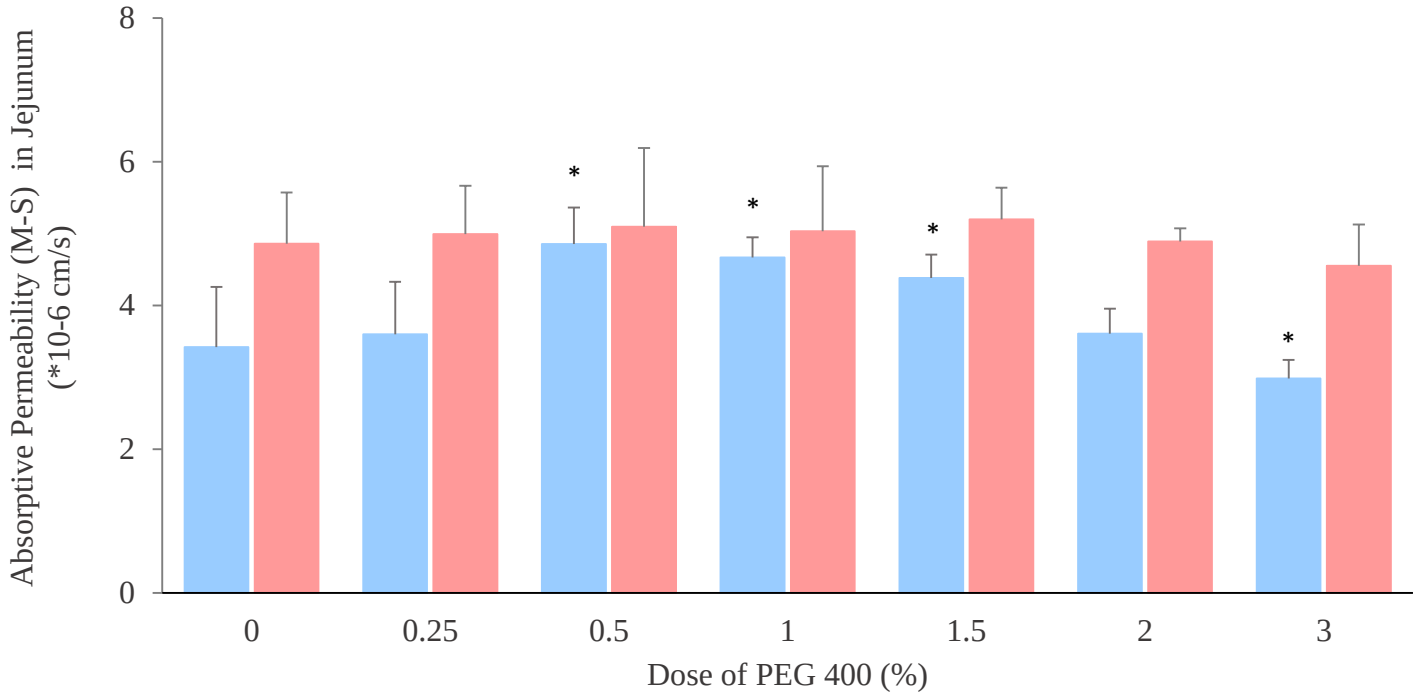


Figure 3.7 Absorptive transport of ranitidine across the jejunum of male (blue) and female (pink) Wistar rats (Mean ± S.D., n=6) in the presence of PEG 400. * Values are statistically different between the control and tested groups at $p<0.05$

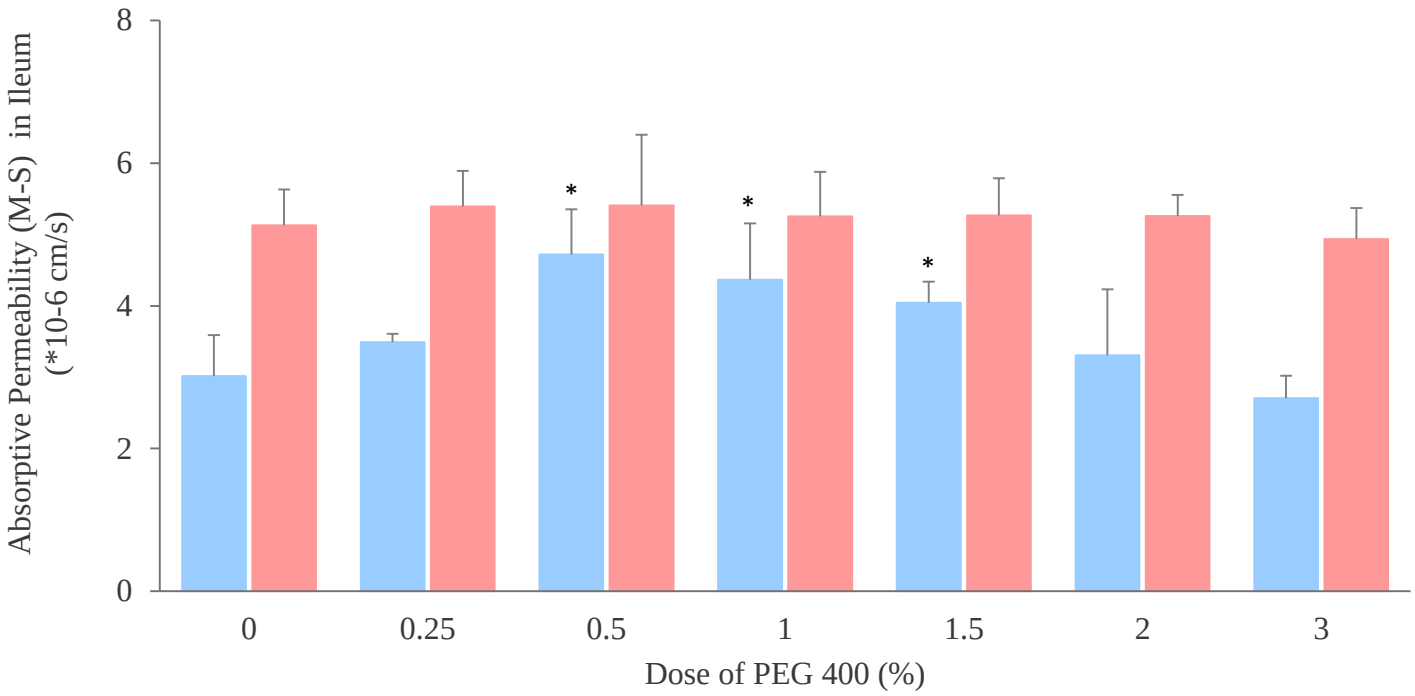


Figure 3.8 Absorptive transport of ranitidine across the ileum of male (blue) and female (pink) Wistar rats (Mean ± S.D., n=6) in the presence of PEG 400. * Values are statistically different between the control and tested groups at $p<0.05$.

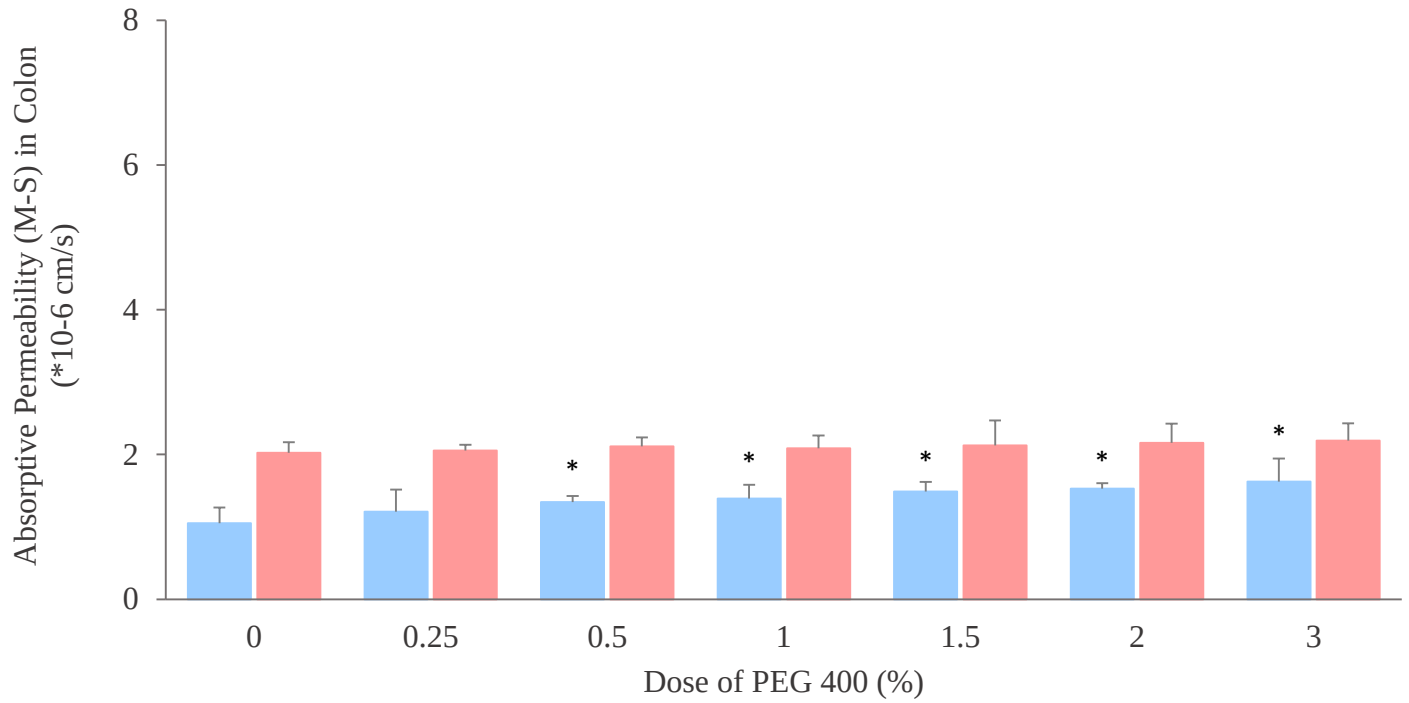


Figure 3.9 Absorptive transport of ranitidine across the colon of male (blue) and female (pink) Wistar rats (Mean ± S.D., n=6) in the presence of PEG 400. * Values are statistically different between the control and tested groups at $p<0.05$.

Furthermore, while PEG 400 enhanced the ranitidine intestinal absorption in male rats, it did not increase in females. In accordance with the data from the previous studies on humans and animals (summarized in Table 3.2), sex-related differences in the jejunal permeability of ranitidine were well matched. It stands that the jejunum should therefore be the subject of further study on this sex-based phenomenon, as compared with other intestinal segments. Moreover, in the jejunum, the differences in ranitidine permeability in males decreased with the amount of PEG 400. The largest differences observed were 49% for doses of 0.5% PEG 400 in the jejunum. Therefore, 0.5% PEG 400 was selected in the following studies.

Table 3.2 Sex differences in the effect of PEG 400 on ranitidine bioavailability in rat study and Ussing chamber study.

Dose of PEG 400 in Ussing chamber Study (%)	Sex Differences in the Segments of Intestine				Sex Differences in Wistar rats study	Dose of PEG 400 in Wistar rats study (mg/kg)
	duodenum	jejunum	Ileum	colon		
0.25						12.9
0.5		√	√	√	√	25.7
0.1		√	√	√	√	51.4
1.5		√	√	√	√	77.1
2				√		102.8
3	√	√		√	√	154.2

(‘√’=obvious sex differences; blank=no sex differences)

3.3.4 The influence of PEG 400 on ranitidine secretion

As shown in Figure 3.10, the presence of 0.5% PEG 400 decreased the secretion of ranitidine in the jejunum, ileum, and colon by 19%, 24% and 31% in male rats ($p<0.05$). However, there were no significant differences found in the secretive transport of ranitidine in the presence of PEG 400 in female rats.

The secretion results, as complementary evidences, strongly indicated that a low dose of PEG 400 could demonstrate a sex-based influence on the efflux of ranitidine in the jejunum, ileum and colon. Since P-gp is the major efflux transporter in the intestinal permeation of ranitidine and PEG 400 is known to inhibit P-gp (Hugger et al., 2002, Shen et al., 2006b), we come to the hypothesis that the intestinal P-gp was the main reason behind this sex-related phenomenon. Thus, to further investigate the role of P-gp, a P-gp inhibitor (CsA) was used, as discussed in the following section.

3.3.5 The effect of PEG 400 on the intestinal permeability of ranitidine in the presence of CsA

The effect of PEG 400 on the absorptive transport of ranitidine in the CsA pre-treated rat intestine is shown in Figure 3.11. Following the pre-incubation period with CsA, ranitidine absorption in the male and female rats was significantly higher ($p<0.05$). However, this effect was notably larger in males compared to female rats in all intestinal divisions. For example, in the jejunum, the absorptive permeability of ranitidine increased by 99% in male rats and in females by 13%. In the ileum, the presence of CsA enhanced the absorption of ranitidine by 74% in males, but only 14% in female rats.

Where vigorously supported, Table 3.3 shows that a marked decrease of ranitidine secretion was obtained with CsA in male intestines only. Additionally, the sex differences of PEG 400's impact on the transport of ranitidine were eliminated once the intestinal tissues were pre-incubated with CsA.

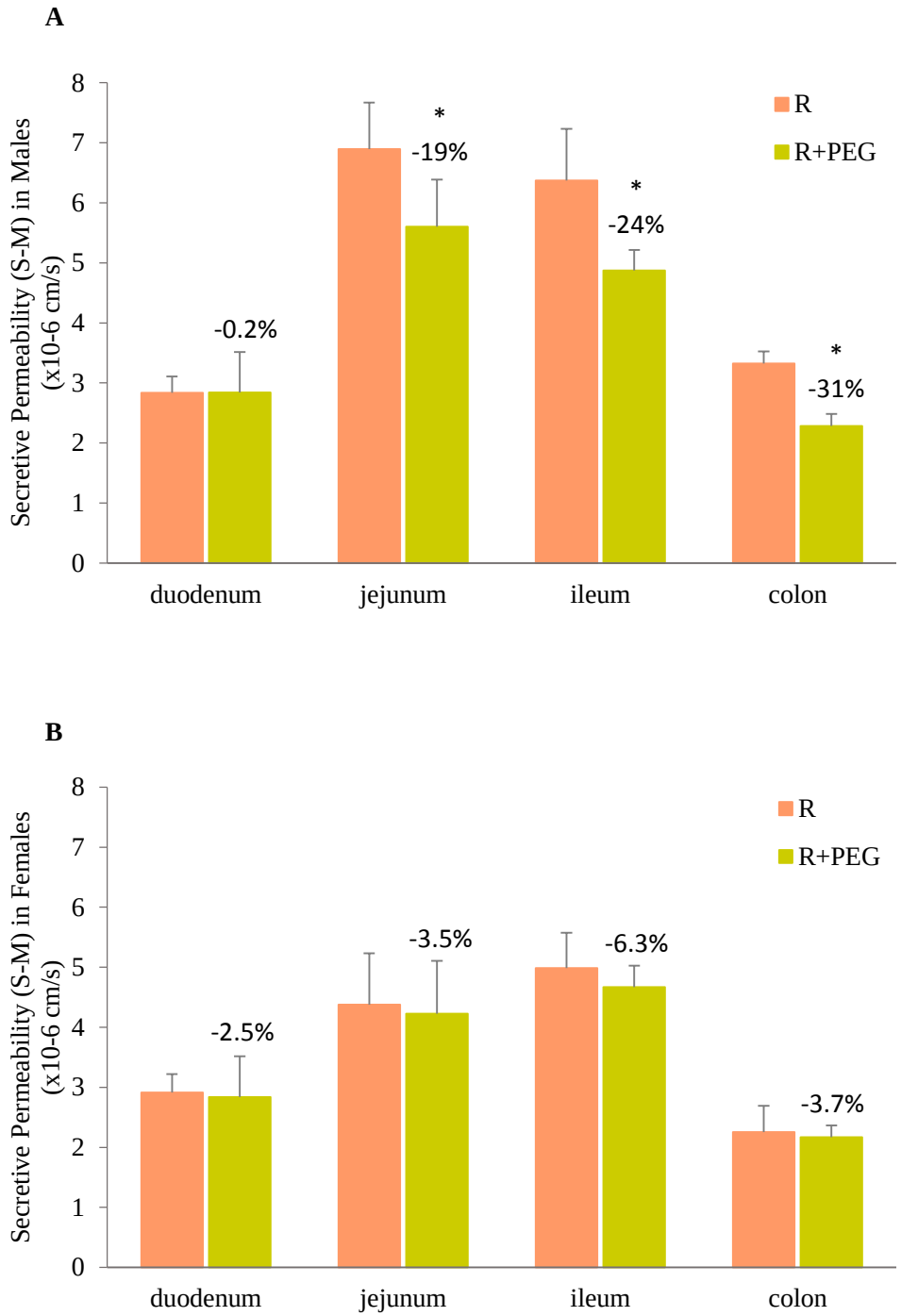


Figure 3.10 Secretive permeability of ranitidine in the absence and presence of PEG 400 in male (A) and female (B) Wistar rats (Mean ± S.D., n=6). * Values are statistically different between the control and PEG group at $p<0.05$.

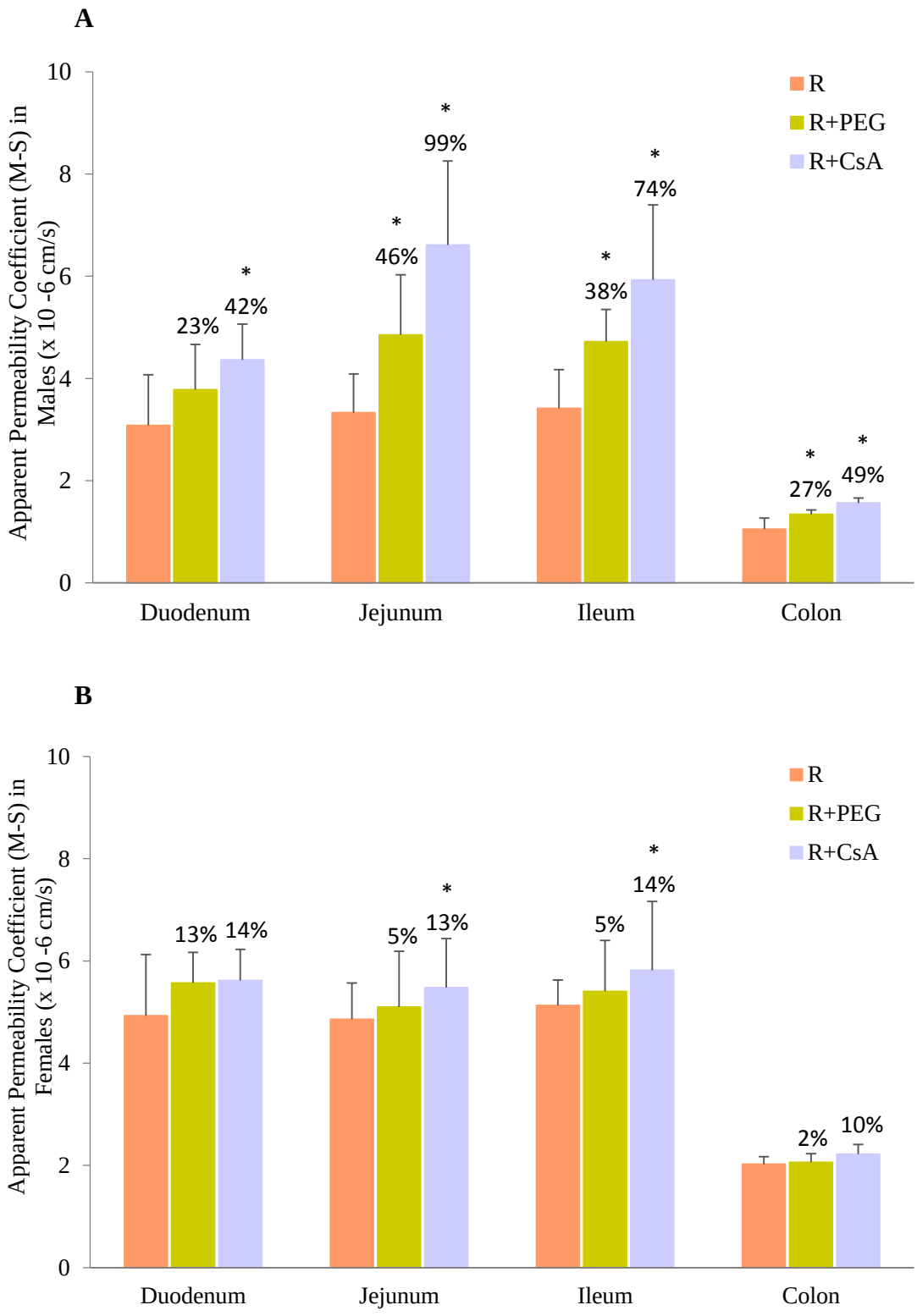


Figure 3.11 Absorptive permeability of ranitidine in the absence and presence of PEG 400 or CsA in male (A) and female (B) Wistar rats (Mean ± S.D., n=6). * Values are statistically different between the control and tested groups at $p<0.05$.

Table 3.3 Efflux ratio of ranitidine in the absence and presence of PEG 400 or CsA in the duodenum, jejunum, ileum and colon of male and female rats (Mean ± S.D., n=6). Ranitidine 3mM, PEG 400 0.5%, CsA 8μM. * Values are statistically different between the control and tested groups at $p<0.5$.

		duodenum	jejunum	ileum	Colon
Males	Ranitidine	1.01±0.36	2.20±0.70	1.96±0.58	3.29±0.83
	Ranitidine + PEG	0.64±0.03 *	1.14±0.22 *	1.05±0.15 *	1.75±0.13 *
	Ranitidine + CsA	0.56±0.11 *	0.64±0.16 *	0.65±0.13 *	1.30±0.12 *
	Ranitidine + PEG + CsA	0.61±0.09 *	0.60±0.11 *	0.67±0.21 *	1.33±0.25 *
Females	Ranitidine	0.55±0.18	0.92±0.25	0.98±0.18	1.36±0.48
	Ranitidine + PEG	0.49±0.14	0.84±0.19	0.88±0.13	1.06±0.09
	Ranitidine + CsA	0.47±0.09	0.71±0.09	0.76±0.17	0.95±0.09
	Ranitidine + PEG + CsA	0.5±0.12	0.73±0.12	0.73±0.15	0.98±0.14

All the results in this study reflect previously published *in vivo* work, which proposed that there was a higher P-gp activity in males compared to females in Chapter 2. The study herein showed that PEG 400 dose-dependently modified the activity of P-gp, which was also shown by Shen *et al*., who demonstrated that the inhibitory effect on P-gp was concentration-dependent across the range of 0.1 to 20% (w/v) (Shen et al., 2006b). Furthermore, the region-related transport of ranitidine in the intestine of rats could also be explained by the reportedly constant increase in the P-gp expression along the small intestine from proximal to distal parts (Yumoto et al., 1999, Tian et al., 2002, Dahan and Amidon, 2009, Kagan et al., 2010). Additionally, sex differences in the P-gp activity might be influencing the effect of PEG 400 on the bioavailability of ranitidine. For example, a study by Ballent et al., provided results that corroborated with a study conducted by Mariana et al., whereby a greater P-gp activity was found in male rats compared to females (Ballent et al., 2012, Mariana et al., 2011). The reason for greater P-gp activity in males compared to females is likely attributed to the sex differences in its own modifying-mechanisms, such as higher P-gp ATPase or ATP levels in the cells of males, and also due to the higher P-gp expression in males' intestine (Gerrard et al., 2004).

The importance of studying sex-based differences has been supported by an increasing body of evidence demonstrating that sex can alter drug efficacy and toxicity profiles (Gandhi et al., 2004); this phenomenon was recently further reported to be influenced by formerly regarded "inert" pharmaceutical excipients. It has been stated that "every cell in our bodies has a sex" (Wizemann and Pardue, 2001), and this was currently endorsed in the study herein. Higher P-gp activity in males was exhibited, which highlights the influence of sex within clinical research and the selection of excipients in the development of oral formulation for drugs, particularly for P-gp substrates. As some excipients are thought to alter the expression of membrane transporters, further work is required to clarify whether

this effect is the same in males and females, and which may have considerable pharmacological and clinical relevance.

3.4 CONCLUSION

In this chapter, the influence of PEG 400 on P-gp-mediated ranitidine transport at the intestinal level showed a sex-dependent manner, in line with the findings in the i*n vivo* study.

Low amounts of PEG 400 increased the intestinal uptake of ranitidine in the jejunum, ileum, and colon in male rats, but not in females using an Ussing chamber. However, no sex-based differences were found in the duodenum, which suggests that this sex-related phenomenon is region-related and dose-dependent. Hence, the PEG 400-mediated modulation of P-gp activity accounted for changes in ranitidine intestinal transport and in the marked differences between male and female subjects, which was also observed in the CsA inhibitory effect. Furthermore, pre-treatment with CsA on rat tissues were shown to inhibit this sex-based effect of PEG 400 on ranitidine intestinal transport. These findings highlight that sex differences should be considered in the use of these excipients, given the aforementioned studies of PEG 400 influencing oral drug bioavailability. Better efficacy and safety outcomes for the use of pharmaceutical excipients could also be potentially obtained with further research on this topic.

CHAPTER 4: Differences in Expression: How does PEG 400 modify the intestinal efflux transporter P-glycoprotein in males and females?

4.1 INTRODUCTION

Our previous studies proposed there is a sex-dependent manner in the interaction between PEG 400 and intestinal P-gp, thus, it was the aim of the work reported here was to investigate the influence of PEG 400 on the intestinal P-gp expression, with the purpose of ascertaining the reason behind the aforementioned sex differences in the effect of PEG 400 on the bioavailability of ranitidine and ampicillin (Ashiru et al., 2008).

In details, the following questions were addressed in this chapter: the protein abundance and mRNA expression of P-gp were studied in four segments of intestine in both male and female rats; and the effect of PEG 400 on the expression of P-gp during a 3-hour period was characterized with Western blotting and real-time reverse-transcription polymerase chain reaction (real time RT-PCR), respectively.

4.1.1 Western blotting

Western blotting (also called protein immunoblotting as an antibody is used to specifically detect its antigen) is a widely accepted analytical technique used to detect specific proteins in the given sample. It uses SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to separate various proteins contained in the given sample. The separated proteins are then transferred or blotted onto a matrix (generally nitrocellulose or PVDF membrane), where they are stained with antibodies (used as a probe) specific to the target protein. By analyzing the location and intensity of the specific reaction, expression details of the target proteins in the given cells or tissue homogenate could be obtained. A schematic representation of Western blotting was shown in Figure 4.1.

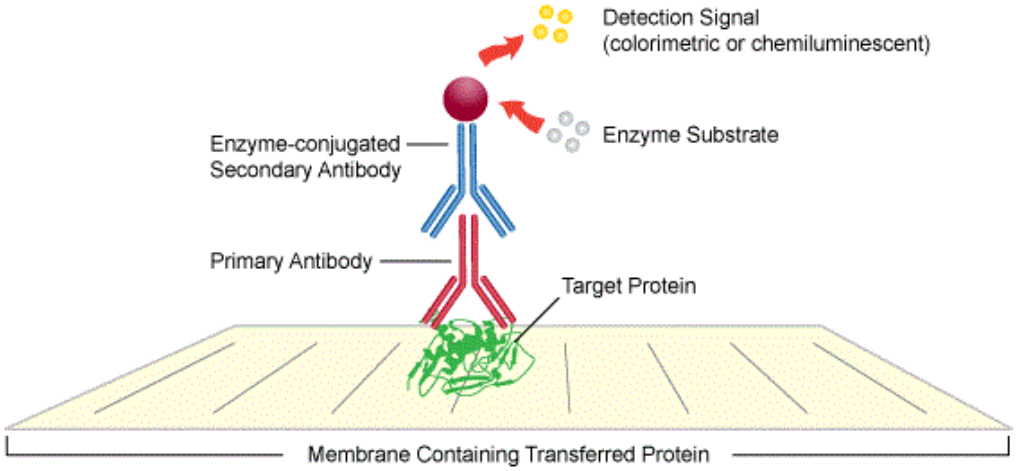


Figure 4.1 The final detection for indirect Western blotting**,** (derived from LeincoTech).

This method is used in the fields of molecular biology, biochemistry, immunogenetics and other molecular biology disciplines. However, Western blotting is not an exact science, particularly in a methodological sense, and obtaining perfect, publication-ready results immediately is not the norm. There are several steps to refine the experimental method and procedures to establish to ensure better results. Details of which are outlined in Table 4.1.

Table 4.1 Factors affecting Western blotting experiment.

Factors	**Variable Characteristic**
Gel	Material, percentage, purity.
Membrane	Toughness, stability, resistance to acids and alkalis.
Transferring	Voltage, time
Primary antibody	Affinity, concentration, incubation time, temperature, pH
Secondary antibody	Cross-reactivity, concentration, incubation time, temperature, pH
Blocking	Concentration, composition, incubation time
Washing	Concentration, composition, volume, number of washes
Detection	Exposure time, sensitivity

Moreover, compared with other targeted protein quantification techniques such as the enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), Western blotting has its own advantages and disadvantages (shown in Table 4.2).

Table 4.2 The advantages and disadvantages of Western blotting.

Advantages	**Disadvantages**
• Immunogenic responses from infectious agents (ex. viruses, bacteria) are hard to find since they are difficult to isolate from patient sample. However, it can be detected by western blotting. • Western blotting utilizes not only antigens, but also antisera as a diagnostic tool. Antisera is widely used in the test for HIV presence. • Compared to ELISA, western blotting has higher specificity. • Since the polyvinylidene difluoride (PVDF) used as membrane in western blotting has a high protein-binding capacity and chemical stability. Some small and unstable protein groups can even be measured. • Among three common enzyme substrates, fluorescent and chemiluminescent create light detectable through X-ray or scanners. This ability enables high levels of sensitivity and quicker processing time.	• A non-intended protein has a slight chance of reacting with the secondary anti-body, resulting in the labeling of an incorrect protein. • Western Blotting is a very delicate process requiring the correct amounts of each component in order for successful identification of the presence of proteins. An imbalance in any step of the procedure may skew the entire process

4.1.2 Real-time qPCR

PCR (reverse transcription-polymerase chain reaction) is the most sensitive technique for mRNA detection and quantitation currently available. Compared to the two other commonly used techniques for quantifying mRNA levels, Northern blot analysis and RNase protection assay, PCR can be used to quantify mRNA levels from much smaller samples. The principle and process of PCR was shown in Figure 4.2.

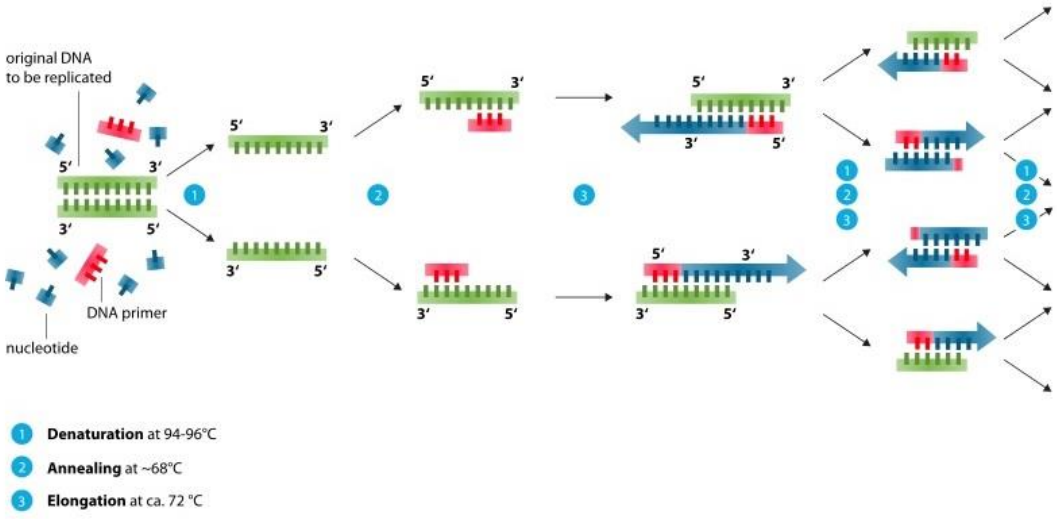


Figure 4.2 The principle and process of PCR.

PCR is conducted on an automated cycler which can heat and cool the tubes with the reaction mixture in a very short time. There are three major steps in a PCR:

1. Denaturation: The double strand melts open to single stranded DNA, all enzymatic reactions stop.

2. Annealing: During this period, the primers are jiggling around. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last for a longer period of time and on the piece of double stranded DNA, the polymerase can attach and start to copy the template. Once there are a few bases built in, the ionic bond is strong between the template and the primer, that it does not break anymore.

3. Elongation: The DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture that are complementary to the template in the 5'-to-3' direction. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template).

Real-time qPCR is a reliable detection and measurement technique of products generated during each cycle of the PCR process. This is directly proportional to the amount of template prior to the start of the PCR process.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Polyethylene glycol 400 (PEG 400) was purchased from Sigma-Aldrich (Poole, UK). Krebs-Bicarbonate Ringer's solution (KBR), pH 7.4, composed of 10mM D-glucose, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 115mM NaCl, 25mM $NaHCO_3$, 0.4Mm KH_2PO_4 and 2.4mM K_2HPO_4 (Clarke, 2009). Lysis buffer (details described in Table 4.3) was freshly prepared with 50mM Tris, 250mM NaCl, 5mM EDTA, 1mM Na_3VO_4, 1mM PMSF, 1% Nonidet P40 and protease inhibitor cocktail in PBS (phosphate-buffered saline). All other chemicals and kits are noted individually in the following methods.

Table 4.3 Components of the tissue protein extraction and cell lysis buffer, (adapted from Dr Vipul Yadav's PhD work).

Ingredient	**Function**	**Quantity for 10mL Buffer**
Tris buffer (10x solution)	Buffering agent	2mL
Sodium chloride	Maintaining ionic strength	146mg
EDTA	Matrix metalloprotease	18mg
Nonidet^{TM} P40 substitute	Lysis of the cell membrane	100L
Sodium azide	Bacterial growth inhibitor	2mg
Sodium orthovanadate	Tyrosine and alkaline	2mg
Phenylmethane sulfonyl fluoride	Serine protease inhibitor	1.8mg
Protease inhibitor cocktail	Mixture of protease	8mL PBS in 1 bottle

4.2.2 Animals

All the animal work was approved by the UCL School of Pharmacy's ethical review committee and was conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act, 1986. Healthy 8-13 week old male and female Wistar rats (Harlan UK Ltd, Oxfordshire, UK) weighing 150-250g were used for excised rat intestine. The rats were housed at controlled temperatures (25℃) and humidity (50-60%) with a constant light-dark cycle of 12h, were provided with food and water and were acclimatized for 7 days. One day before the experiments, the rats were fasted overnight and housed individually in metabolic cages.

On the day of the experiment, each rat was dosed with 26mg/kg PEG 400 using an oral gavage syringe. Subsequently, rats were killed after 15min, 30min, 60min, 90min, 120min or 180min using a CO_2 euthanasia chamber, and their intestines were immediately collected and kept in ice-cold KBR solution.

4.2.3 Mucosal tissue preparation

The intestines were cut into 4 segments: the duodenum (1cm from the ligament of Treitz); the jejunum (10cm from the ligament of Treitz); the ileum (1cm from the cecum) and the colon, which were then washed with ice-cold KBR solution. Roughly 2cm pieces in length from the mid part of the duodenum, proximal part of the jejunum, the distal to mid part of ileum and the descending colon were opened along their mesenteric border. The tissues were washed gently with KBR solution to remove the intestinal contents. To obtain the mucosal tissue, tissue pieces were placed on an ice-cold glass plate and the serosa layer was gently squeezed out and divided into aliquots for determination of P-gp protein content and mRNA expression in detail below.

4.2.4 Detection of P-gp protein level in intestinal segments by Western blotting

4.2.4.1 Sample preparation

The mucosal tissues (about 60mg) were cut into small pieces and homogenized in 3mL lysis buffer at 10,00rpm for 20s on ice with a T18 digital ULTRA-TURRAX® (IKA, Wilmington, USA). The tissue homogenates were incubated at 4°C for 2h, then centrifuged at 10,000rpm for 10min. The supernatants were collected and stored in aliquots at -20°C.

4.2.4.2 Sample preparation

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4.2.4.3 P-gp Protein Level Analysis

25μg of total protein of each sample was suspended in LDS (lithium dodecyl sulfate) sample loading buffer (Invitrogen, Carlsbad, CA) and denatured for 10min at 70°C. As a molecular weight marker, 5μL Sharp Pre-Stained protein standard (Invitrogen) was loaded on each gel.

Protein in the samples were separated by electrophoresis in a NuPAGE™ Novex™ 4–12% Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane with XCell SureLock™ Mini-Cell Electrophoresis System (Invitrogen) according to the manufacturer's instructions. Nitrocellulose membranes were blocked with 3% BSA (bovine serum albumin) in TBS-T (0.1% Tween 20 in tris-buffered saline) and incubated for 1h at room temperature. For detection of P-glycoprotein (P-gp) and

reference protein, blots were incubated for 1h at room temperature with the respective primary antibodies diluted in 3% BSA in TBS-T: mouse monoclonal anti-P-gp (C-219 3:200; Enzo Life Science, Exeter, UK) and anti-β-actin mouse monoclonal antibody (1:2000; Sigma-Aldrich, Poole, UK). Bound antibodies were detected with affinity-purified rabbit anti-mouse IgG coupled to horseradish peroxidase (secondary antibody; Sigma) diluted 1:5000 in 3% BSA in TBS-T.

After 1h incubation with the secondary antibody conjugated with horseradish peroxidase, protein bands were visualized by chemiluminescence detection with Pierce™ ECL Western Blotting Substrate (ThermoFisher), subsequently were photographed with a ChemiDoc XRS camera (Bio-Rad, Hertfordshire, UK). Detection of bands of P-gp and reference protein was performed using the Image Lab™ software (Bio-Rad). For a calculation of the relative P-gp contents in the different samples, the reference protein was individually set to 1, and the intensity of P-gp was set relative to it.

4.2.5 Measurement of P-gp mRNA expression in intestinal divisions by real-time reverse-transcription polymerase chain reaction

4.2.5.1 RNA isolation

All pieces of mucosal tissue (for segment definitions, please refer to Tissue Preparation) were cut and kept in RNA later® Stabilization Solution (Thermofisher). Total RNA of each piece was isolated with The TRIzol® (Table 4.4) and purified with PureLink® RNA Mini Kit (Thermofisher) according to the manufacturer's instructions (briefly described in Figure 4.3). RNA concentration was measured with Nanodrop 2000 (Thermofisher).

Table 4.4 TRIzol reagent composition, function and optimized concentrations.

Ingredient	**Function**	**Concentration**
Phenol	Extraction and purification of nucleic acid	38%
Guanidine thiocyanate	Denaturation of proteins (such as those that strongly bind nucleic acids or those that degrade RNA)	0.8M
Ammonium thiocyanate	Denaturation of proteins	0.4M
Sodium acetate	Maintain pH and provides the salt necessary for RNA precipitation.	0.1M
Glycerol	Carrier for nucleic acids	5%

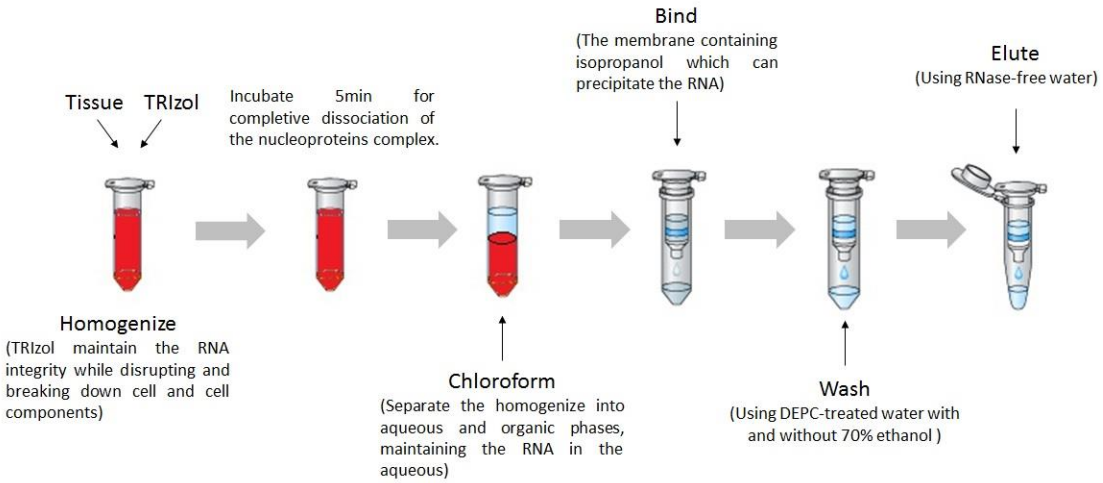


Figure 4.3 The process of RNA isolation with TRIzol-chloroform method.

4.2.5.2 RNA level analysis

Subsequently, the quantification of the target RNA was conducted as followed: 1 mg total RNA of each sample was reverse transcribed using the iScript™ cDNA Sythesis Kit (Bio-Rad). To quantify the amount of P-gp mRNA (*mdr1a* and *mdr1b)*, real-time PCR was performed on the 7500 Real Time PCR System (Applied Biosystems, Thermofisher) using the method described in MacLean's study (MacLean et al., 2008). Briefly, 50μL PCR reaction contained 25μL of PowerUp™ SYBR Green PCR Master Mix (Thermofisher), 500nM each of forward and reverse primers, and 1μg of cDNA. Anti-beta actin was used for normalization and amplification of 1μg cDNA, respectively. Real-time PCR was carried out in 96 well PCR plates (Thermofisher). The amplification program for all genes consisted of one pre-incubation cycle at 95°C with a 10min hold, followed by 45 amplification cycles with denaturation at 95°C with a 10s hold, an annealing temperature of 50°C with a 10s hold, and an extension at 72°C with a 10s hold. Amplification was followed by a melting curve analysis, which ran for one cycle with denaturation at 95°C with a 1s hold, annealing at 65°C with a 15s hold, and melting at 95°C with a 1s hold. Distilled water was included as a negative control in each run to access specificity of primers and possible contaminants.

Primers (shown in Table 4.5) were designed by primer-BLAST searching with publicly available sequence information of the GeneBank of the National Center for Biotechnology Information (NCBI) and purchased from Eurofins (Eurofins Genomics, Germany).

Table 4.5 Primers used for the analysis of P-gp gene expression by real-time qPCR

Gene		Primer (5' – 3')	Amplicon (bp)	Genebank Accession
mdr1a	Forward	CACCATCCAGAACGCAGACT	139	NM_133401
	Reverse	ACATCTCGCATGGTCACAGTT		
mdr1b	Forward	AACGCAGACTTGATCGTGGT	144	NM_012623
	Reverse	AGCACCTCAAATACTCCCAGC		
anti-beta actin	Forward	GCAGGAGTACGATGAGTCCG	74	NM_031144
	Reverse	ACGCAGCTCAGTAACAGTCC		

4.2.5.3 Data analysis

Relative expression of *mdr1a* and *mdr1b* mRNA in different intestinal segments were calculated using 7500 software (version 2.0.6, Thermofisher). The average of the threshold cycle (Ct) values for tested genes (*mdr1a* and *mdr1b*) and the internal control (anti-beta actin) was taken, and then the differences between Ct values for tested genes and internal control (ΔCt) were calculated for all the experimental samples.

4.2.6 Statistical analysis

The experiments were performed at least six times and data were expressed as mean ± standard deviation (S.D.). Significant differences among groups were analysed by one-way ANOVA and three-way ANOVA using IBM SPSS Statistics 19 (SPSS Inc., Illinois, USA). A minimum p value of 0.05 was used as a significance level for the tests. Also, the relationship between P-gp protein levels and mRNA expression (both *mdr1a* and *mdr1b*) was investigated using Pearson product-moment correlation coefficient (r).

4.3 RESULTS AND DISCUSSION

4.3.1 P-gp protein abundance in different intestinal segments

To obtain a general picture of the expression profile of P-gp along the intestinal tract, the amount of P-gp protein was quantified in the duodenum, jejunum, ileum and colon in male and female rats. As can be seen from Figure 4.4, there is a constant increase in the P-gp content along the intestine from proximal to distal parts (duodenum < jejunum < ileum < colon), with the trend being more obvious in males compared to female rats.

A progressive increase in P-gp protein abundance and activity from the proximal to the distal small intestine (Yumoto et al., 1999, Tian et al., 2002, Dahan and Amidon, 2009, Kagan et al., 2010), with the highest P-gp levels appearing in the colon (Fojo et al., 1987, Fricker et al., 1996) has previously been reported in rodents. In contrast to rodents, the P-gp contents in the intestine of human subjects is different. A recent study where the absolute P-gp content from six organ donors was quantified using LC-MC/MS (Drozdzik et al., 2014) showed a 3-fold higher P-gp level in the distal ileum compared to the duodenum or the proximal jejunum, alongside notably lower P-gp levels in the colon. Regardless of the different techniques used in the above studies, the influence of location on the protein content was similar in rats and humans for the small intestine, but not the colon. The lower P-gp level and related efflux in the proximal (in contrast to the distal) small intestine explains why the proximal end is the ideal absorption site for drugs which are P-gp substrates. It also explains how compounds which reduce the gastrointestinal motility, such as sodium acid pyrophosphate, mannitol and sorbitol, can significantly increase the oral absorption of concomitantly administered P-gp substrate drugs (Adkin et al., 1995a, Chen et al., 2007).

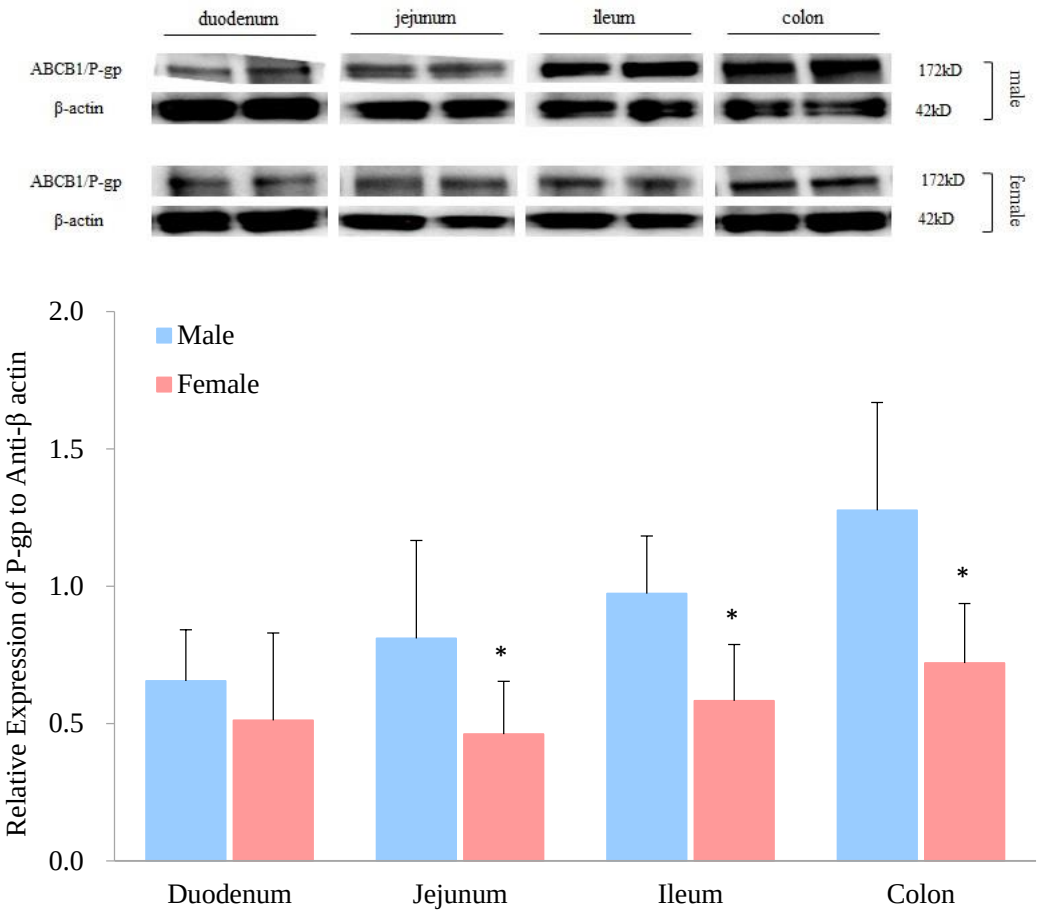


Figure 4.4 Relative quantitation of P-gp protein abundance in selected intestinal segments (duodenum, jejunum, ileum and colon). The levels of protein are normalized to anti-β actin. * Values are statistically different between the male and female groups at $p<0.05$.

Figure 4.4 also shows a considerable influence of an animal's sex on the intestinal P-gp level. While the P-gp abundance was similar in male and female duodenums ($p>0.05$), it differed markedly in other intestinal segments. Specifically, abundance was about 40% higher in the male jejunum, ileum and colon ($p<0.05$). This reflects previous reports of higher enterocyte P-gp content in men's small intestine compared to women's (Schuetz et al., 1995a, Potter et al., 2004), and a lack of difference in the upper duodenum (Ungell et al., 1992, Tozaki et al., 1997). As far as the colon is concerned, there is limited literature on sex-related P-gp protein abundance or mRNA expression in rodents and humans. In contrast to our studies, MacLean et al (2008) reported no significant sex-related difference in the intestinal P-gp expression along the whole intestine of male and female rats (MacLean et al., 2008). Such a difference between our studies and those of MacLean et al could have been due to the fasted/fed status of the rats, which were fasted in our studies but fed in MacLean's study, as well as high inter- and intra-variability.

The presence of food was indeed reported to modify the P-gp expression in the intestine. Grapefruit juice, as an example, was reportedly prohibited in the clinical studies due to its inhibition on P-gp, increasing the cyclosporine (P-gp substrate) AUC ranging from 20% to 60% (Huang et al., 2004). The ingredient furanocoumarins in grapefruit juice was later confirmed to be the main reason for its P-gp inhibition, as conducted on Caco-2 cells (Paine et al., 2008). Aside from the inhibitory effects, P-gp expression could also be induced in the presence of food. For example, garlic extracts have been demonstrated to decrease drug exposure for saquinavir (P-gp substrate), owing to its induction of P-gp expression in the human intestine and liver (Hajda et al., 2010).

4.3.2 Influence of PEG 400 on the P-gp protein contents

The influence of orally administered PEG 400 on P-gp protein level in the jejunum is shown in Figure 4.7. Similar results are found in duodenum, ileum and colon (Figure 4.6, Figure 4.8 and Figure 4.9). It can be seen that PEG 400 administration reduced P-gp protein levels in a time- and sex- dependent manner. In male rats, the reduction in P-gp protein levels peaked at 60 min post PEG dosing, after which, P-gp levels seem to recover to their original values. In contrast, in female rats, P-gp levels decreased to a much lower extent compared to the control values (when PEG was not administered), such that no statistical differences were found. There was a general trend of decreasing P-gp levels, however, this trend continued for a longer time, i.e. until the end measurement at 180 min.

Reduction of P-gp levels by PEG 400 in the rats reflects a previous report where PEG 400 reduced P-gp protein abundance and enhanced the uptake of Rho-123 (a P-gp substrate) into Caco-2 cells (Hodaei et al., 2015). The mechanisms underlying the reduction in P-gp levels was unclear, and the authors suggested that the alteration of fluidity of the epithelial membranes by PEG 400 was regarded as the principal reason for the inhibition of P-gp activity and enhancement in the Rho-123 uptake. An earlier report showed that PEG 400 and other excipients such as Tween-80 and F-68 blocked the binding sites of P-gp and altered the membrane fluidity (Li et al., 2011). Although, there is still controversy over whether P-gp-inhibiting excipients increase or decrease the fluidity of the epithelial membranes (Dudeja et al., 1995, Rege et al., 2002), it is known that P-gp is highly sensitive to the lipid environment of the cell membrane (Regev et al., 1999, Ferte, 2000). Therefore, any disturbance in this environment by excipients can possibly result in changes in the secondary and tertiary structures of the P-gp protein. It is feasible that PEG 400, containing oxyethylene groups, can intercalate in the lipid phase of the membrane, thereby changing the latter's fluidity (Hugger et al., 2002). It has also been suggested that the alkyl and unsaturated C-C bonds in the chemical structure of PEG derivatives may influence the function of P-gp (Shen et al., 2006a). On the other hand, the modulation of excipients on the nuclear receptors was also considered as another main reason behind the phenomenon, given that transporter

protein abundance is generally regulated by these nuclear receptors, such as pregnane receptor in rodents (PXR), retinoic acid receptor (RAR) and farnesoid receptor (FXR) (Yoshikawa et al., 2002, Lee et al., 2006a). For instance, mRNA expression of P-gp was strongly predicted by the mRNA levels of PXR, whose signaling pathway for intestinal efflux transporter was likely in a constantly activated physiological state (Drozdzik et al., 2014). All the hypothesis are summarized in Figure 4.5.

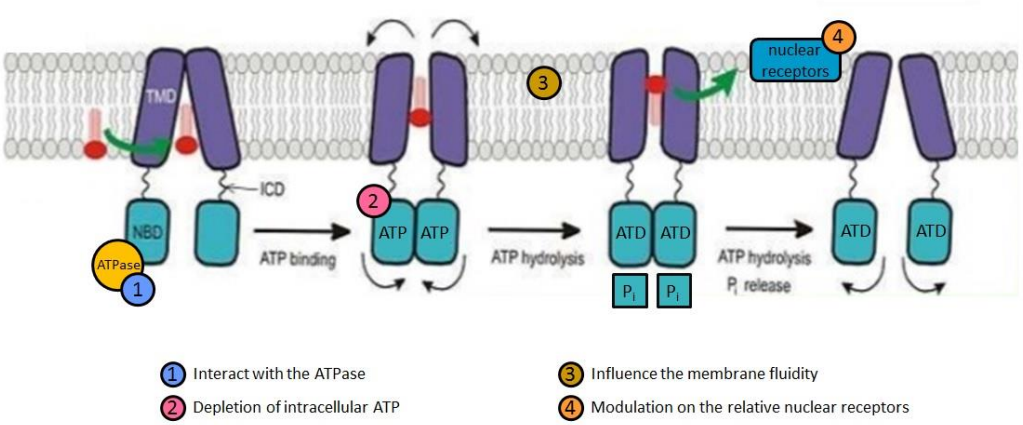
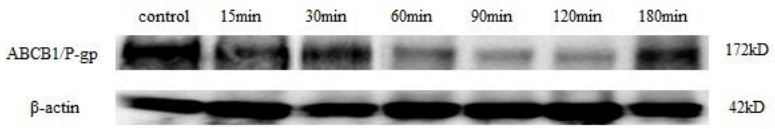


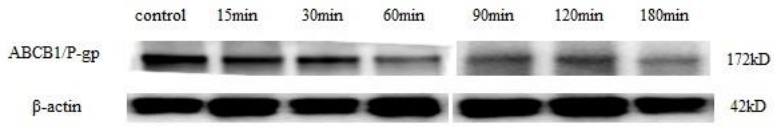
Figure 4.5 The mechanisms on the interaction between PEG 400 and intestinal P-glycoprotein.

While the above studies shows that P-gp modulation by excipients is well-known, we report, for the first time, an influence of animal's sex. The influence of PEG 400 on the P-gp abundance in female rats was quite different to that in the males. Firstly, PEG 400 administration had a longer-lasting effect such that the P-gp protein content continuously decreased over the 3hr duration. Secondly, the magnitude of reduction in P-gp levels was lower in females. The reason behind this sex-based phenomenon is unknown, but the rat intestinal P-gp seems to be less sensitive to PEG 400 in females than in males. It is possible that the intestinal membrane was more stable to changes in fluidity and for that the epithelial renewal was slower in females compared to males. To further understand the time- and sex- dependent influence of PEG 400 on P-gp levels, P-gp related mRNA expression was measured and is discussed below.

Male-Jejunum



Female-Jejunum



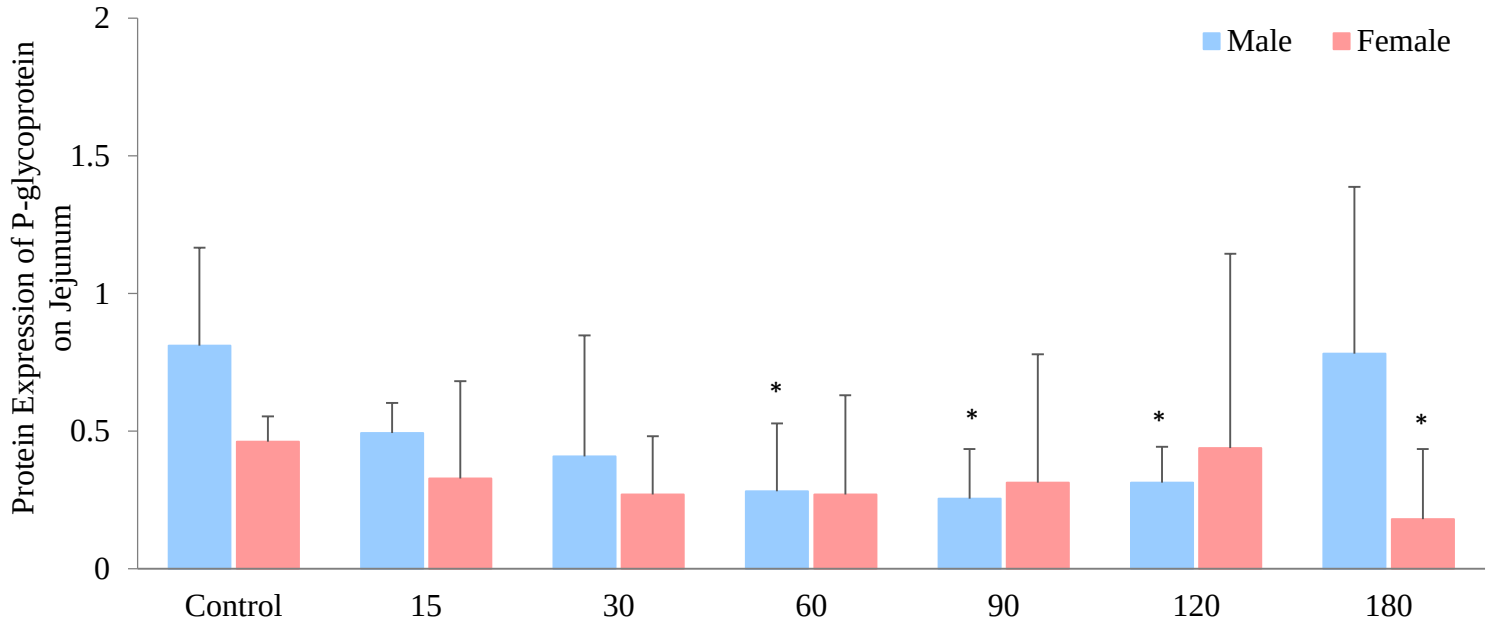
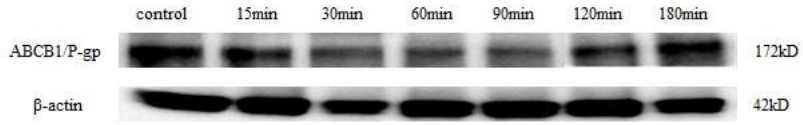


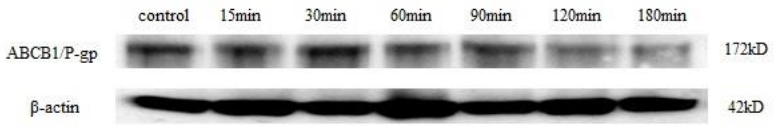
Figure 4.6 The effect of PEG 400 on relative quantitation of P-gp protein expression in jejunum over 3h.

The levels of protein are normalized to anti-β actin. * Values are statistically different between the control and PEG groups at $p<0.05$.

Male-Duodenum



Female-Duodenum



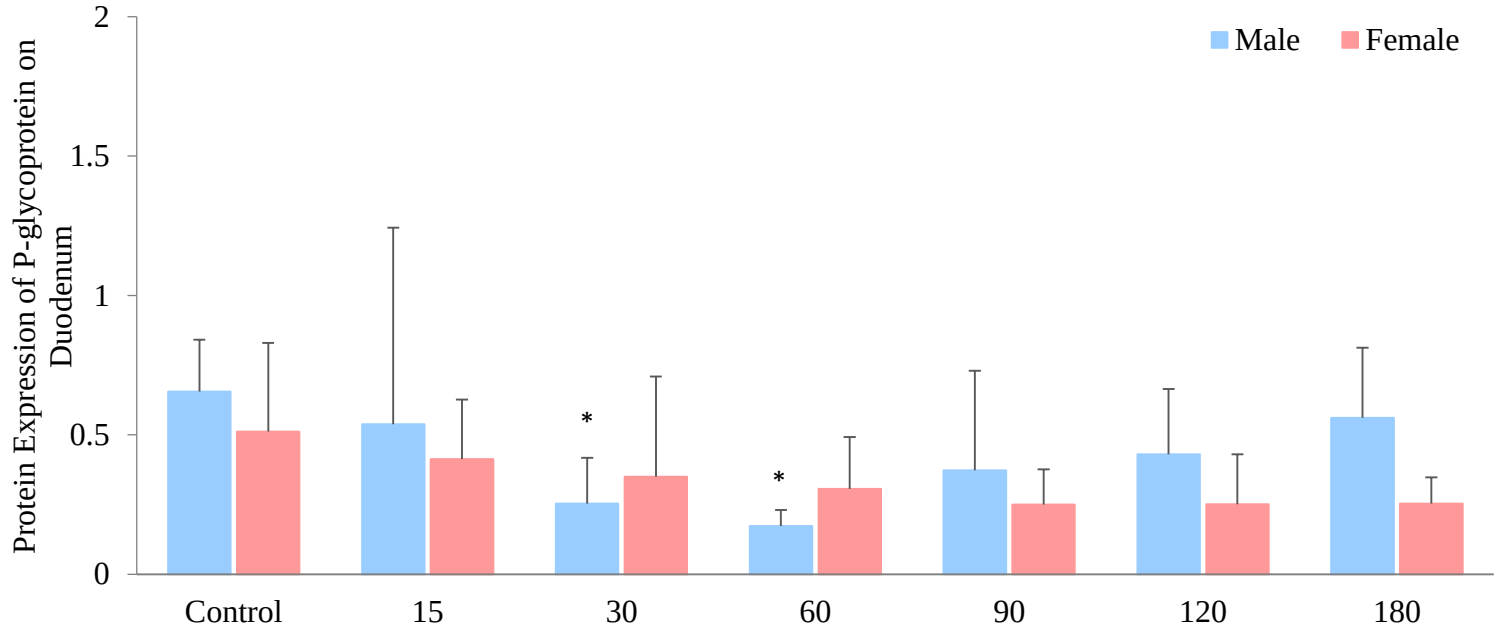


Figure 4.7 The effect of PEG 400 on relative quantitation of P-gp protein expression in duodenum over 3h.

The levels of protein are normalized to anti-β actin. * Values are statistically different between the control and PEG groups at $p<0.05$.

Male-Ileum

Female-Ileum

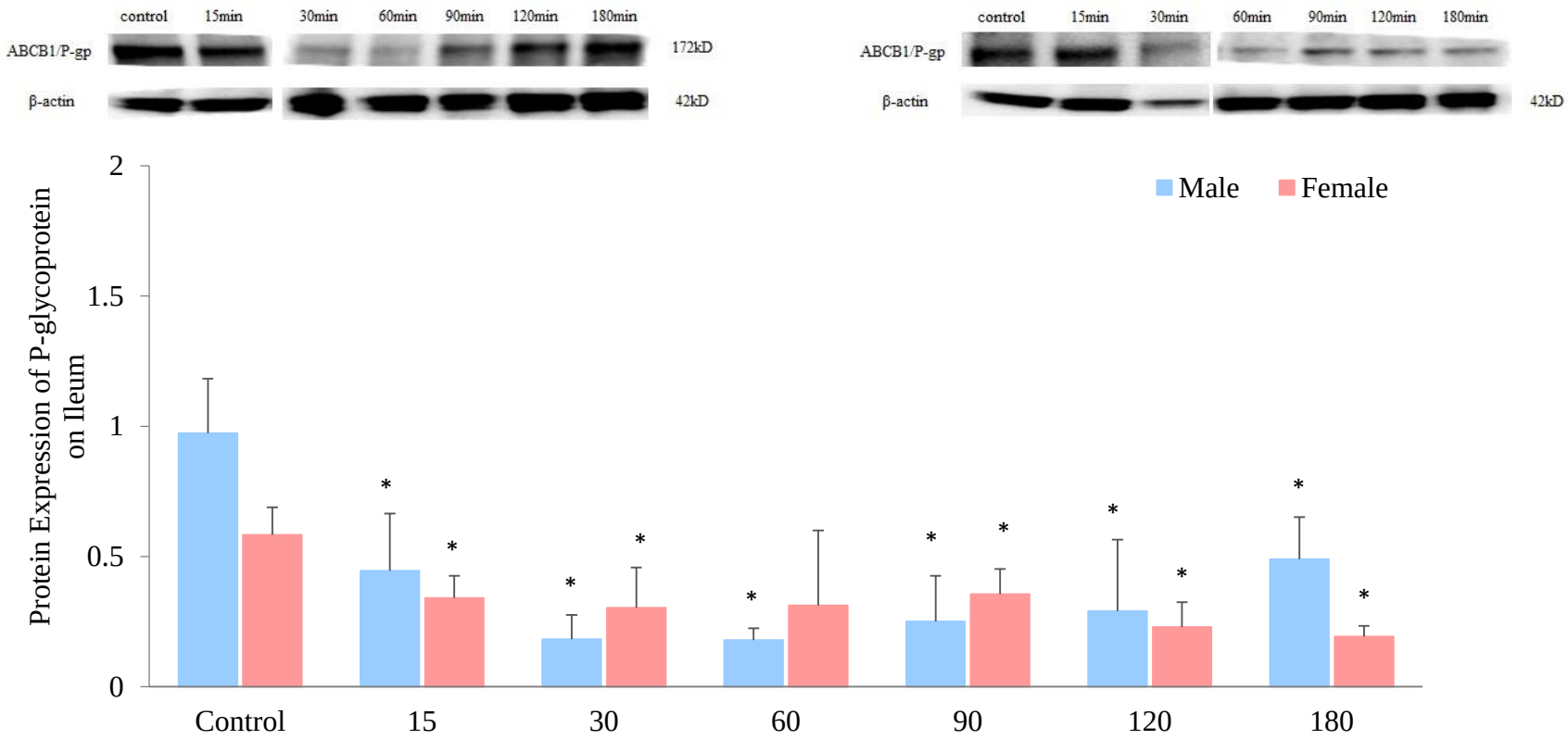


Figure 4.8 The effect of PEG 400 on relative quantitation of P-gp protein expression in ileum over 3h.

The levels of protein are normalized to anti-β actin. * Values are statistically different between the control and PEG groups at $p<0.05$.

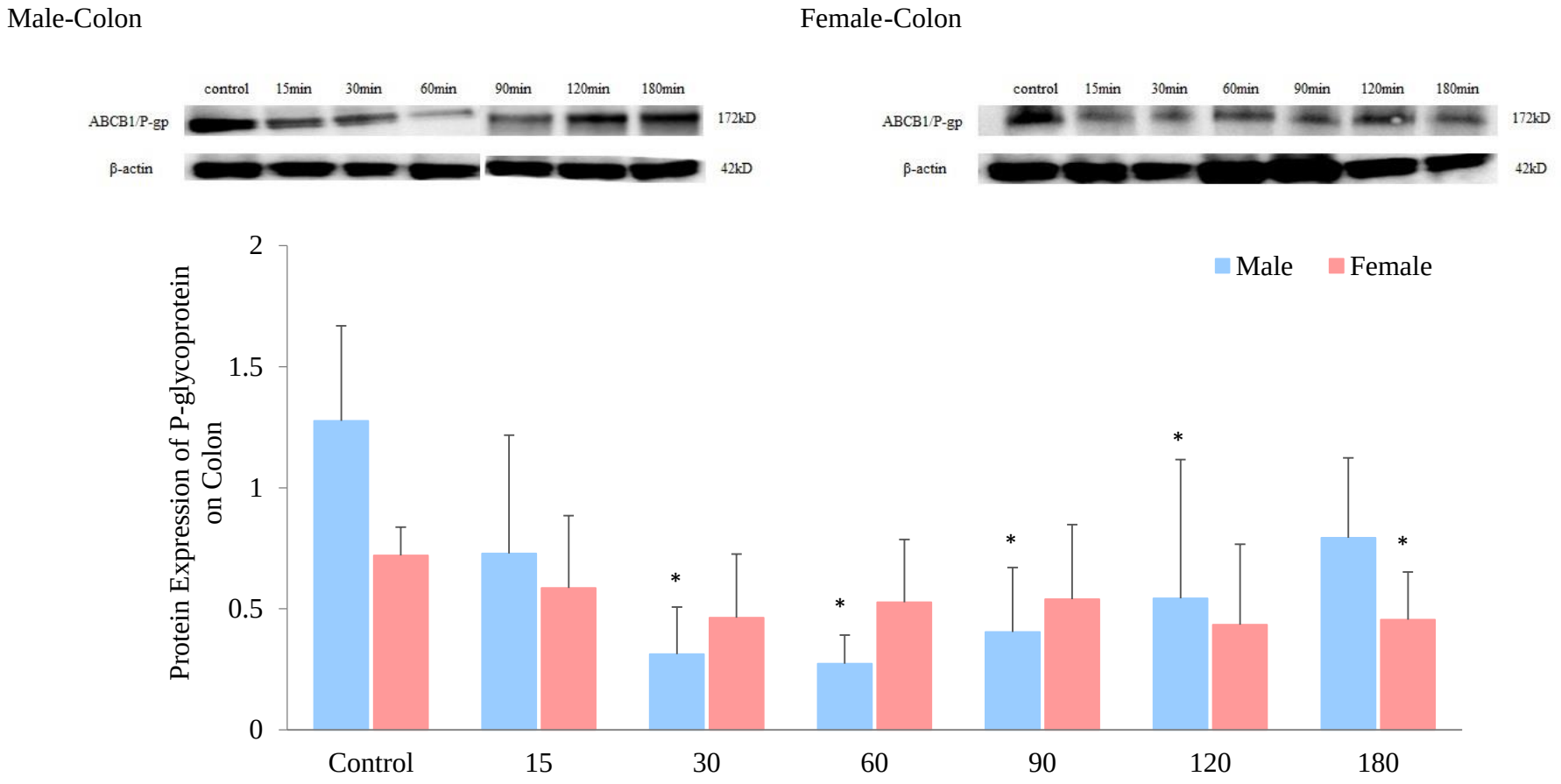


Figure 4.9 The effect of PEG 400 on relative quantitation of P-gp protein expression in colon over 3 hours.

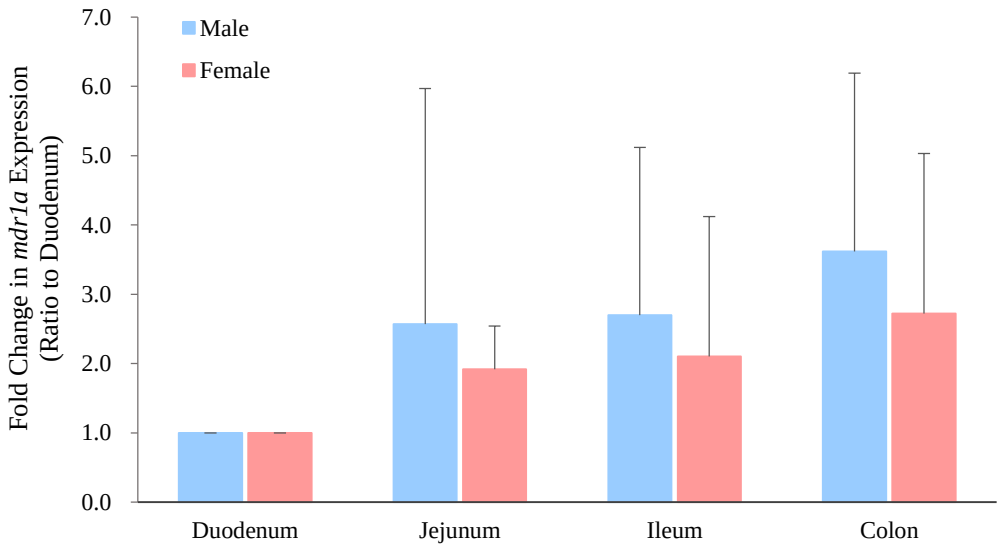
The levels of protein are normalized to anti-β actin. * Values are statistically different between the control and PEG groups at $p<0.05$

4.3.3 P-gp mRNA (*mdr1a* and *mdr1b*) expression in the absence and presence of PEG 400.

The control (i.e. without PEG administration) gene expression of P-gp, namely *mdr1a* and *mdr1b*, is shown in Figure 4.10. In both male and female rats, the *mdr1a* expression of P-gp increased from the proximal to the distal end of the intestinal tract, whereas no obvious trend was observed for the *mdr1b* expression along the intestine. When an animal's sex was considered, *mdr1a* expression consistently lower in females except the duodenal segment, while any trend in *mdr1b* expression was once more lacking (shown in Figure 4.11).

Following oral administration of PEG 400, the *mdr1a* expression in the male rat intestine decreased in the first 1 to 2 h and then constantly increased returning to the control within 3h of PEG administration. In contrast, *mdr1a* expression in female jejunum kept decreasing followed the PEG 400 PEG 400 administration and *mdr1a* contents had not return to control levels by the end of the study (shown in Figure 4.13 for the jejunum). Similar results were found in the duodenum, ileum and colon (graphed in Figure 4.12, Figure 4.14 and Figure 4.15). The jejunal *mdr1b* expression in both males and females fluctuated with time after PEG 400 dosing, and no trend could be seen.

A



B

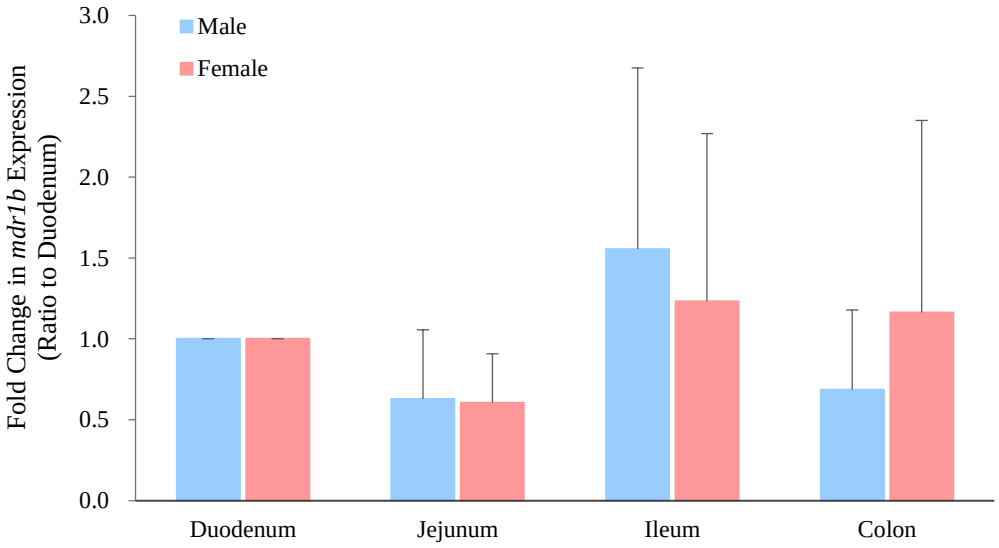
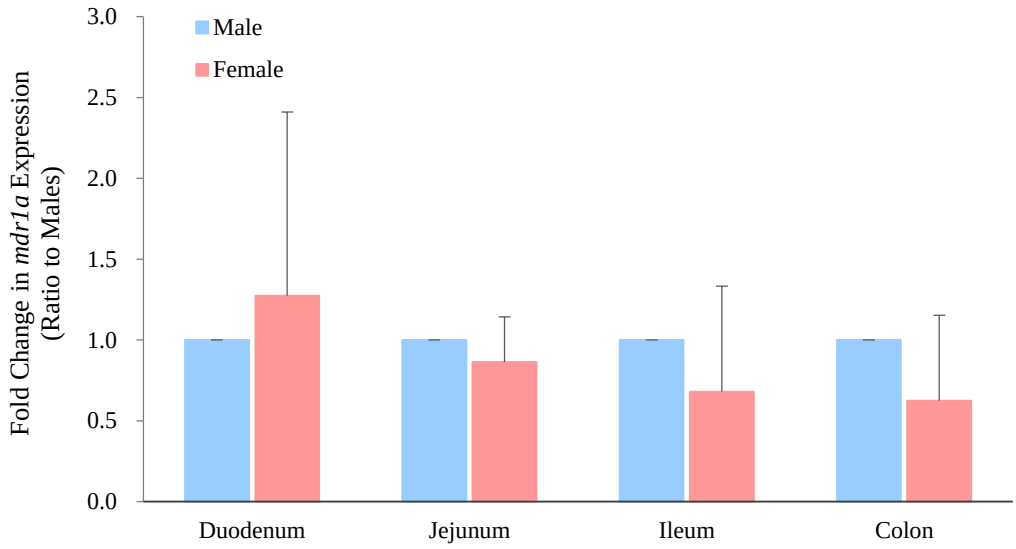


Figure 4.10 Comparison of *mdr1a* (A) and *mdr1b* (B) mRNA expression in different intestinal segments of rats. The level of mRNA is normalized to anti-β actin. Expression intensities of duodenum were individually set to 1 and the intensities of jejunum, ileum and colon were relative to it.

A



B

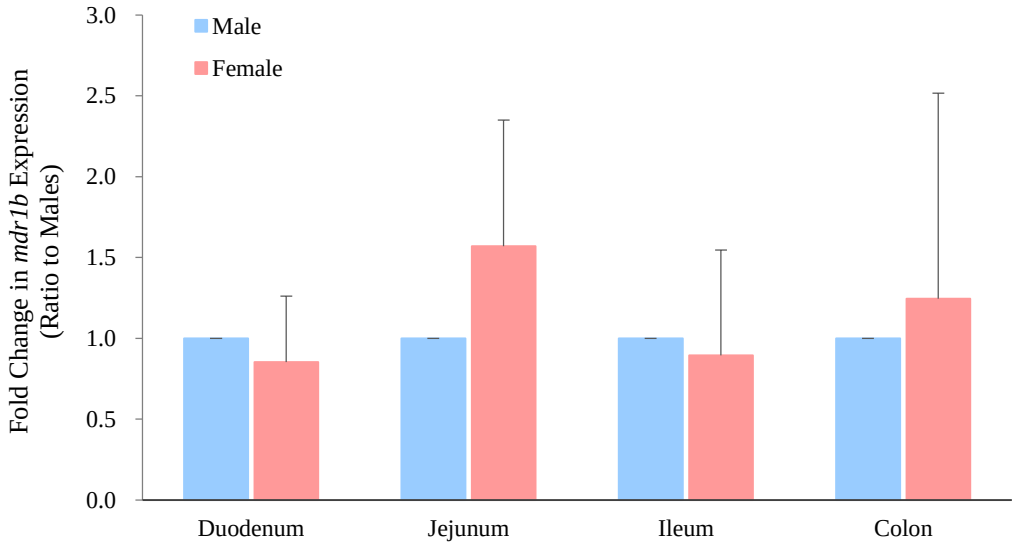


Figure 4.11 Comparison of *mdr1a* (A) and *mdr1b* (B) mRNA expression in the male and female rats. The level of mRNA is normalized to anti-β actin. Expression intensities in male rats were individually set to 1 and the intensities in females were relative to it.

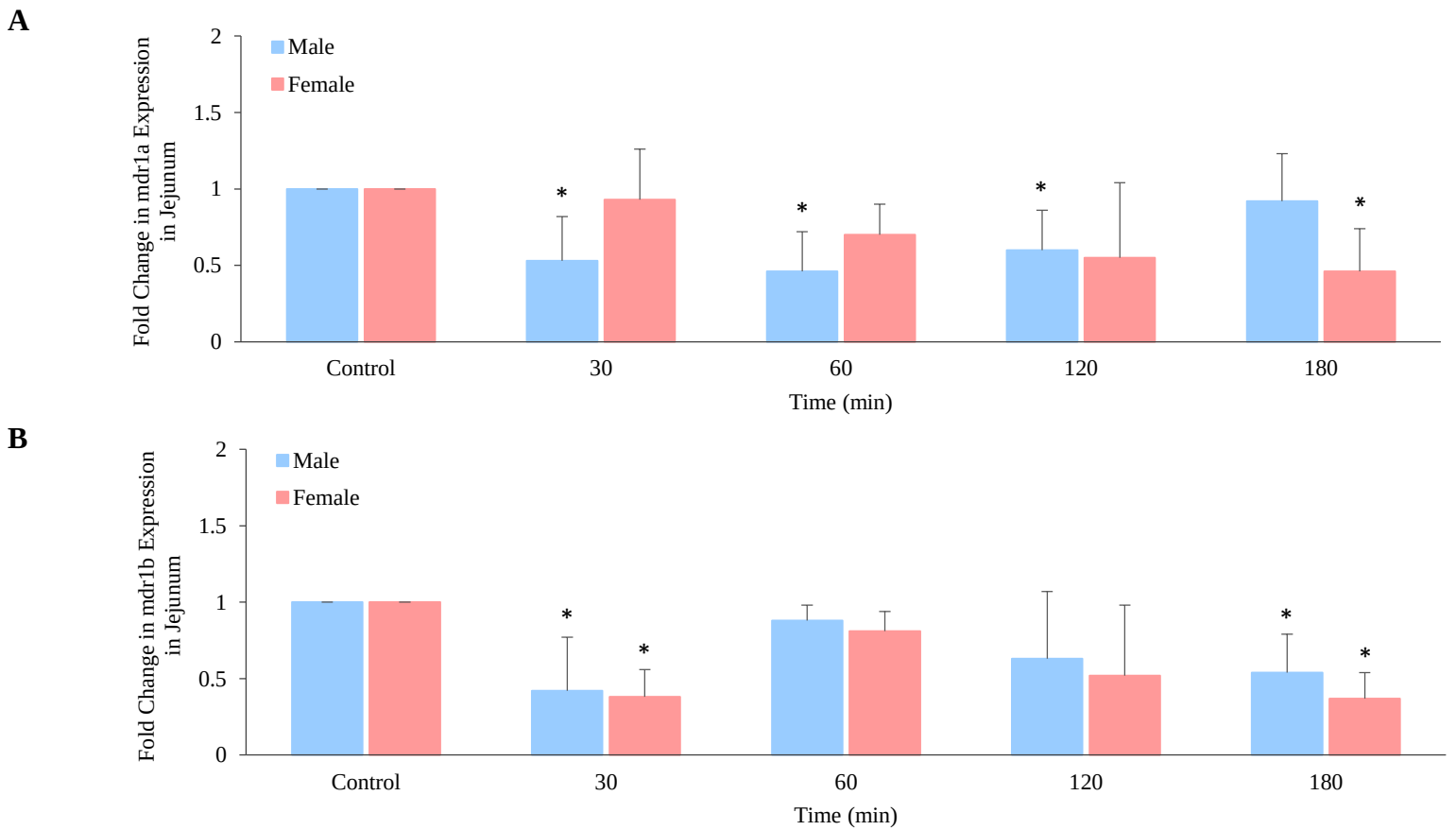


Figure 4.12 Fold changes in *mdr1a* (A) and *mdr1b* (B) mRNA expression in jejunum of rats after 3h administration with PEG 400.

* Values are statistically different between the control and PEG groups at $p<0.05$.

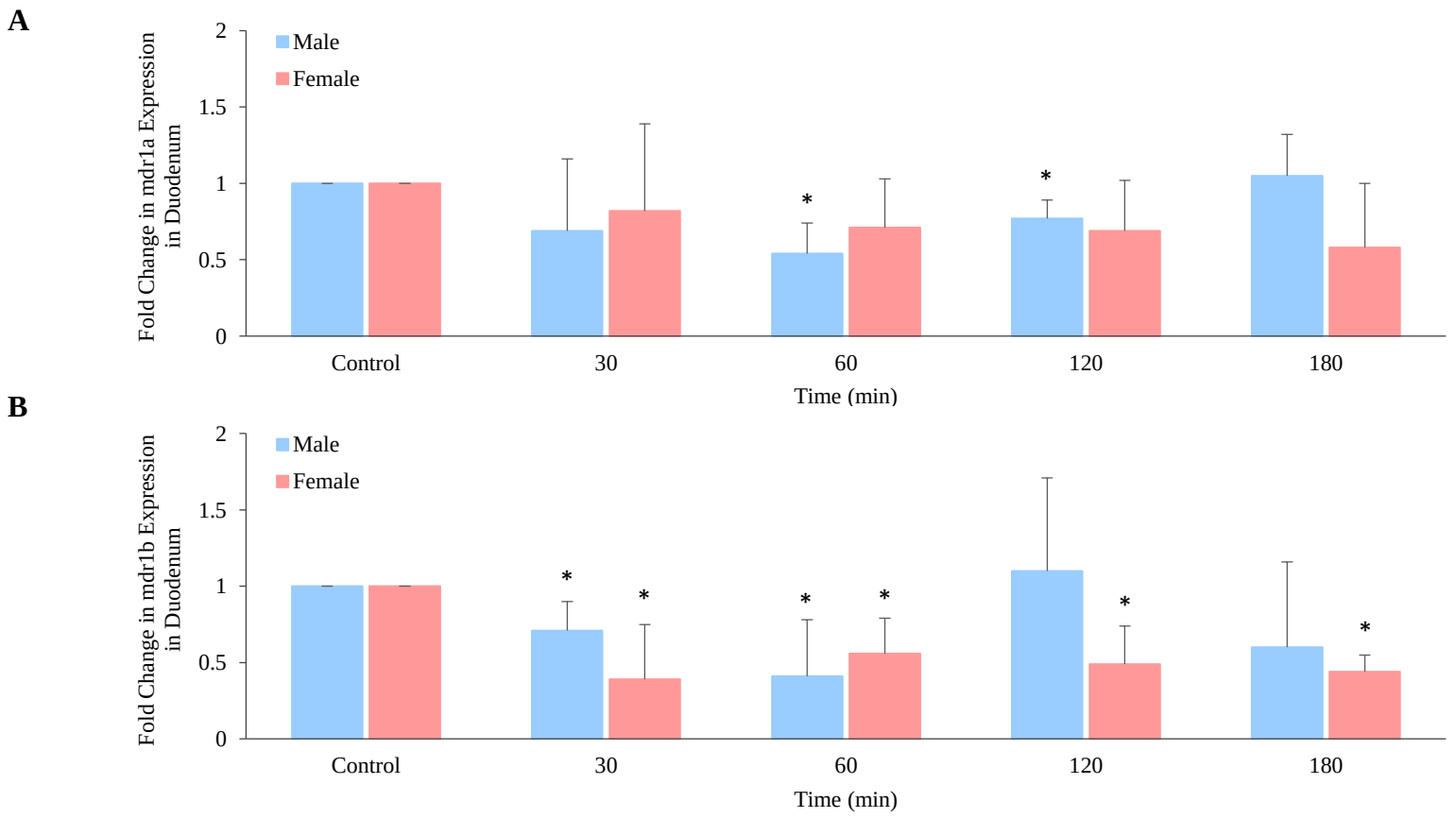


Figure 4.13 Fold changes in mdr1a (A) and mdr1b (B) mRNA expression in duodenum of rats after 3h administration with PEG 400.

* Values are statistically different between the control and PEG groups at $p<0.05$.

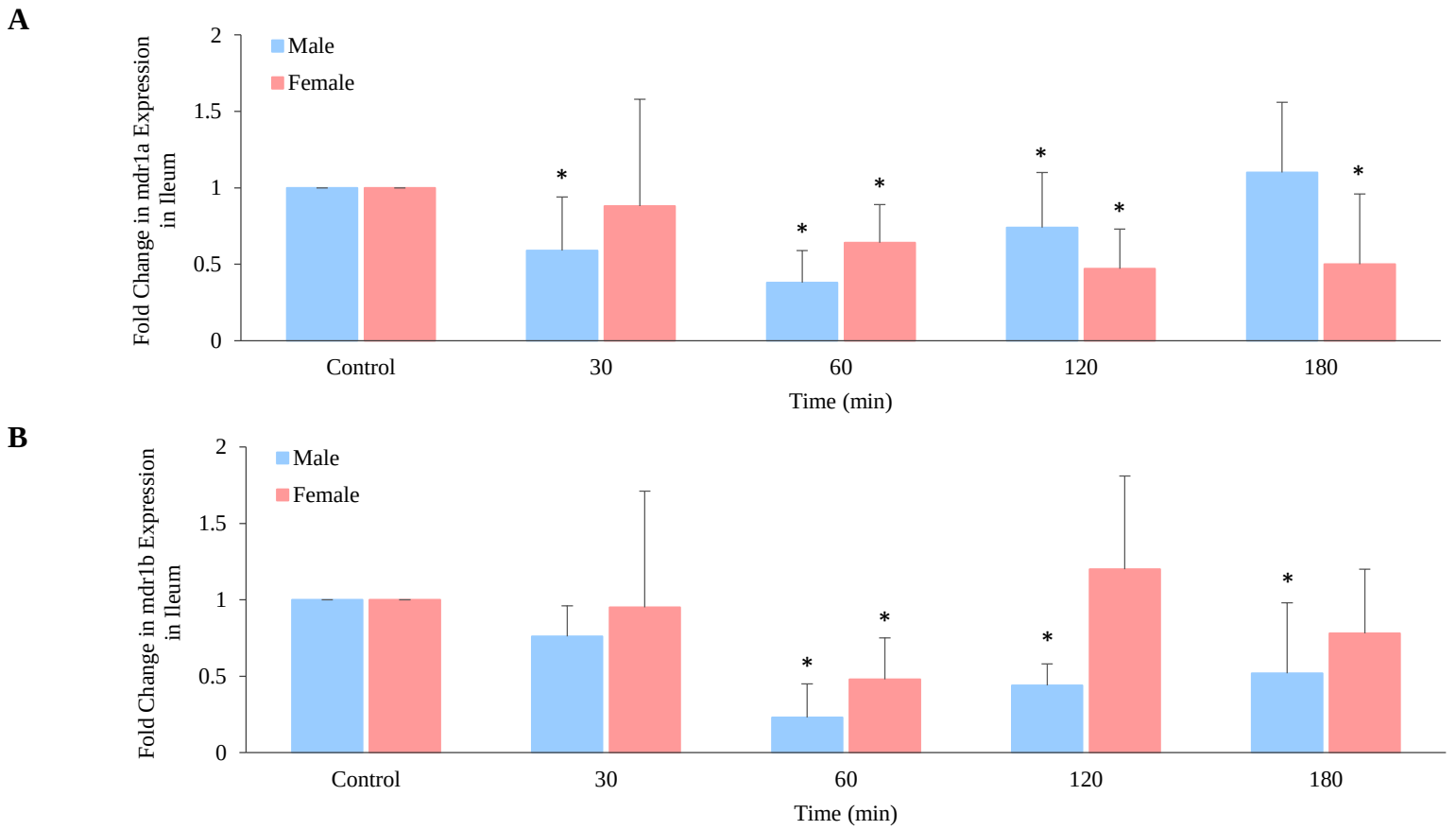


Figure 4.14 Fold changes in *mdr1a* (A) and *mdr1b* (B) mRNA expression in ileum of rats after 3h administration with PEG 400.

* Values are statistically different between the control and PEG groups at $p<0.05$.

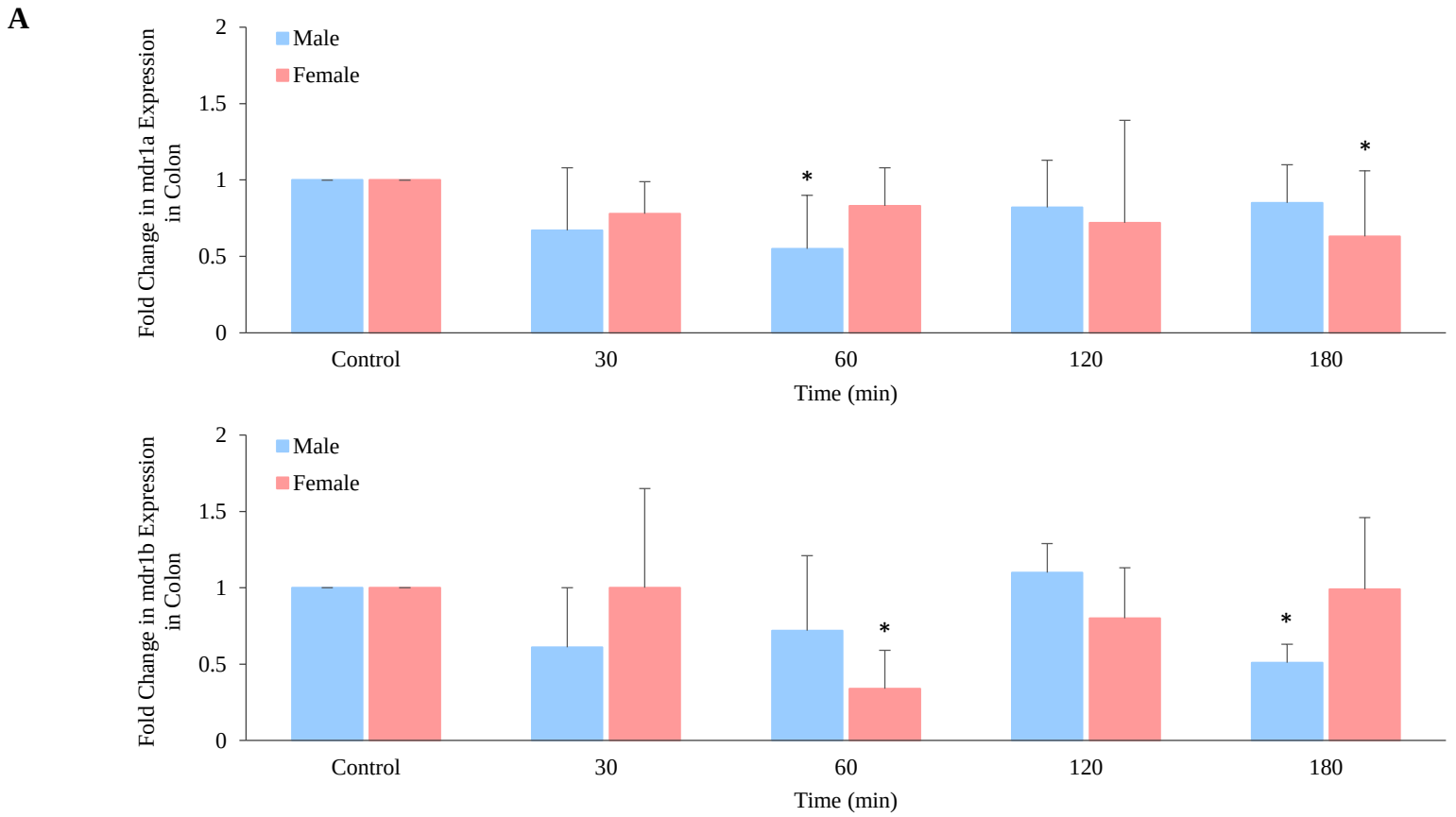


Figure 4.15 Fold changes in *mdr1a* (A) and *mdr1b* (B) mRNA expression in colon of rats after 3h administration with PEG 400.

* Values are statistically different between the control and PEG groups at $p<0.05$.

It can be seen from Figure 4.16 and Table 4.6, there is a strong positive correlation between *mdr1a* (but not *mdr1b*) expression and P-gp protein abundance, r=0.78, n=336, r<0.001, with high levels of P-gp protein associated with high levels of *mdr1a* expression.

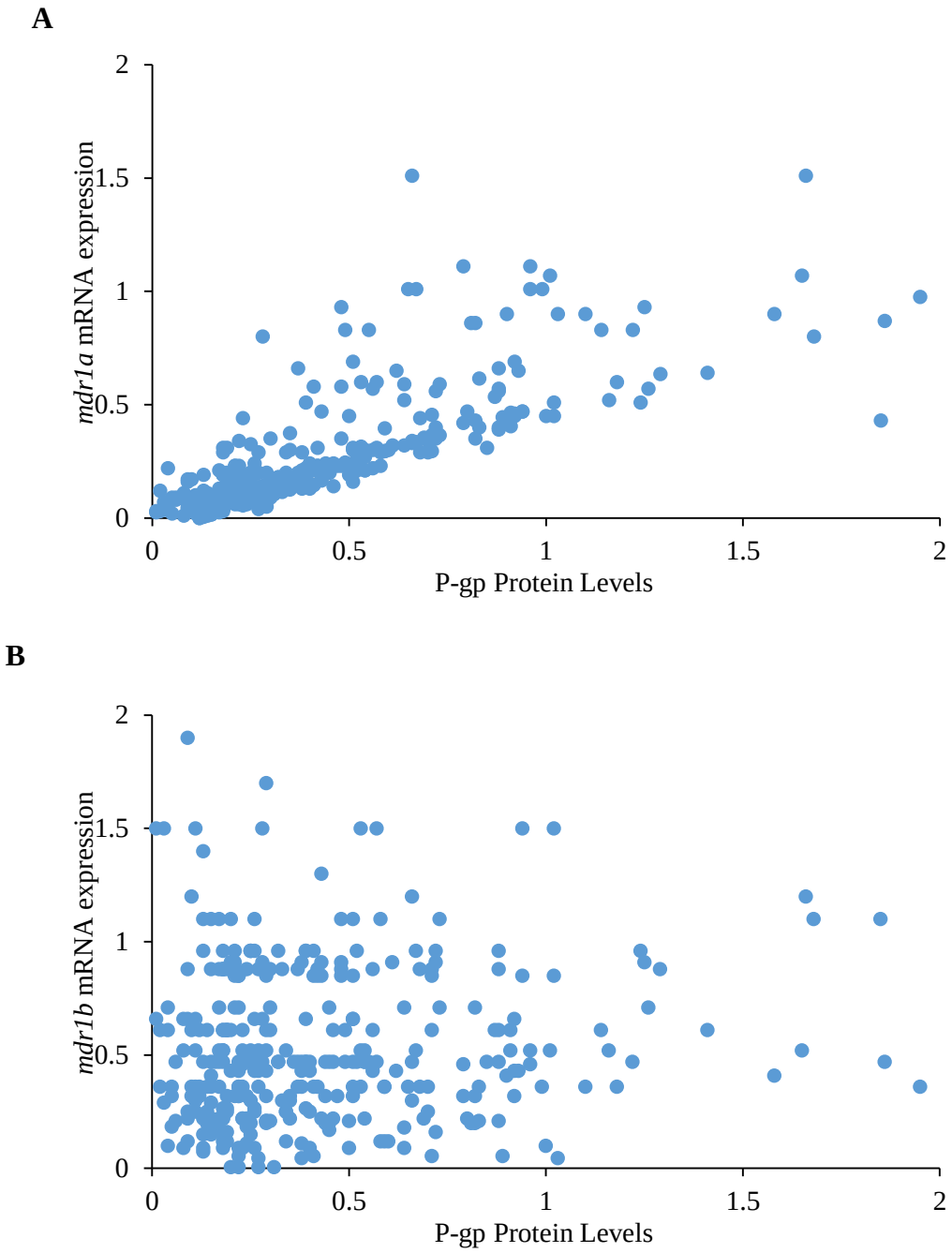


Figure 4.16 Correlation coefficients between P-gp protein levels and its related *mdr1a* (A) and *mdr1b* (B) mRNA expression.

This positive correlation in *mdr1a* expression and P-gp protein level in the rats reflects similar positive correlation between gene expression and absolute intestinal P-gp protein level in humans (Drozdzik et al., 2014). Having said that, the literature about the relationship P-gp and its mRNA levels is contradictory. It has been suggested that the predictive power of transcript analysis must be investigated on a gene-by-gene and/or a case-by-case basis, and that mRNA expression data should only be used as supportive information regarding protein levels. Nevertheless, from our work, we can conclude a greater usefulness of *mdr1a* compared to *mdr1b* for predicting P-gp protein levels.

Table 4.6 Correlation coefficient (r) from Pearson correlation analysis between P-gp protein levels and its related mRNA expression (*mdr1a* and *mdr1b*).

Groups	P-gp protein level	*mdr1a* expression	*mdr1b* expression
P-gp protein level	——	0.785 **	0.095
mdr1a expression		——	0.083
mdr1b expression			——

** $p < 0.001$ (2-tailed).

This work also raises several new aspects that could be discussed. To begin with, the colonic P-gp protein abundance differed from rodents and human subjects, possibly due to different protein quantification techniques used in these studies. It may be worth confirming the absolute protein level of P-gp in rodents with the same method LC-MC/MS, and aid in the characteristic of membrane transporters in animal models related to human. Also, it could help to elucidate the semi-quantitative methods, such as Western blotting, applied in this study. A second consideration is that fast-release dosage forms would be a better choice in the oral formulation development of P-gp substrates, owing to lower P-gp content in the

proximal small intestine disregarding both species and sex of the subjects. Furthermore, inclusion of "active" pharmaceutical excipients, such as PEG 400, are likely to benefit oral formulations containing P-gp substrates that are BCS Class IV drugs (poor solubility and poor permeability), as PEG 400 could both improve the solubility and permeability of co-formulated drugs. Finally, the unexpected sex differences demonstrated in this study highlights a potential safety concern, requiring a greater consideration in the selection and use of pharmaceutical excipients for oral formulation development. These findings further underline the role of sex in the drug pharmacokinetics and clinical studies.

4.4 CONCLUSION

The work in this chapter further elaborates on the sex-related influence of the purportedly inactive excipient PEG 400 on the absorption of drug which are P-gp substrates. This influence can now be attributed to sex differences in the intestinal P-gp protein levels and its related mRNA expression. We confirmed female had lower levels of P-gp protein as well as mRNA expression. In addition, female intestinal P-gp levels seem to be less sensitive (in terms of extent and timing) to oral administration of PEG 400. The reason for and mechanism underlying this sex-related difference is still unknown, but needs to be clarified in future work.

CHAPTER 5: Exploring Excipients: Are there other excipients that exhibit differences in drug absorption due to our sex?

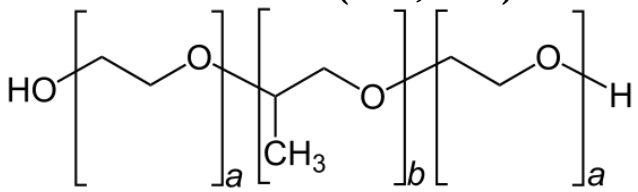
5.1 INTRODUCTION

In the previous study, we have thoroughly investigated the reason behind the unexpected sex-dependent influence of PEG 400 on the bioavailability of ranitidine. The results showed that this effect was attributed to its sex-specific reduction on the P-gp activity, protein level and mRNA expression. Pharmaceutical excipients are assumed to be safe for long-term therapy as they have been used for many years in pharmaceutical formulations and are approved for use by responsible government bodies. Thus, our findings precisely highlight the importance of excipient selection in formulation strategy.

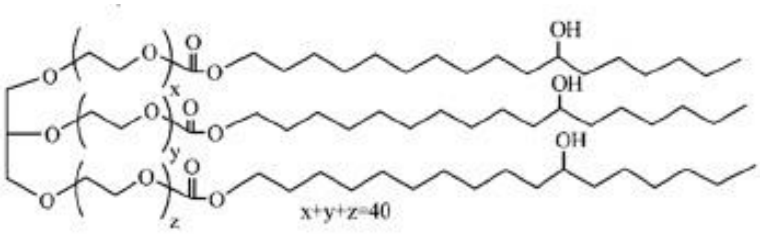
Apart from PEG 400, other solubilizing agents, such as Cremophor RH 40, Poloxamer 188, Tween 80 and Span 20 (chemical structures shown in Figure 5.1), were also reported to interact with membrane transporters, however, knowledge about whether their influences were sex-specific is very limited. Moreover, the transport inhibitory activity has been found to be below the critical micelle concentration (CMC) of the surfactant. Not only does the transport inhibitory activity relate to the hydrophile-lipophile balance (HLB) of surfactants, but also their concentrations (Varma and Panchagnula, 2005). In addition, the excipients Cremophor RH 40, Poloxamer 188, Tween 80 and Span 20 were commonly used in the commercial products at a wide range of concentrations.

Consequently, the purpose on this chapter was to investigate the effect of these formerly considered “inert” excipients on the bioavailability of ranitidine in male and female rats, with the aim of exposing any sex-associated or dose-dependent or manners in the interplay between these excipients and drug absorption. The work herein was conducted in two phases.

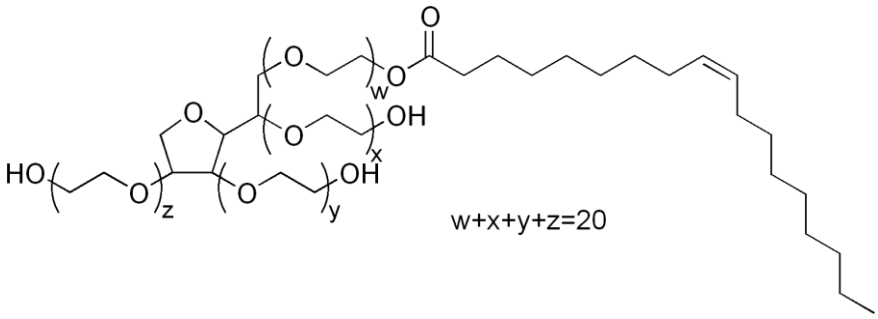
Poloxamer 188 (a=80; b=27)



Cremophor RH 40



Tween 80



Span 20

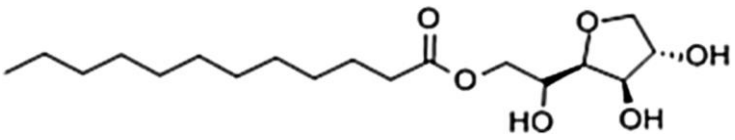


Figure 5.1 Chemical structures of tested excipients.

Phase I: Screening the influence of other excipients (Cremophor RH 40, Poloxamer 188, Tween 80 and Span 20) on the drug absorption in male and female rats, with the aim of establishing if these impacts are sex-dependent as the effect of PEG 400. Ranitidine was selected as the model drug in this study. Also, only one dose of each excipient was selected based on the ratio of their molecular weight to PEG 400, which was calculated using the format as followed:

$$\text{Concentration (Excipient)} = \frac{Concentration(PEG\ 400) \times Molecular\ Weight(Excipient)}{Molecular\ Weight\ (PEG\ 400)}$$

The study was designed as followed:

- The influence of Cremophor RH 40, Poloxamer 188, Tween 80 and Span 20 on the *in vivo* bioavailability of ranitidine in rats.
- The effect of Cremophor RH 40, Poloxamer 188, Tween 80 and Span 20 on the *in vitro* intestinal transport of ranitidine in rats, using Ussing chamber system.
- The impact of Cremophor RH 40, Poloxamer 188, Tween 80 and Span 20 on the P-gp protein abundance and mRNA expression, with Western blotting and real-time RT-PCR respectively.

If sex differences were shown in the influence of the tested excipients on ranitidine bioavailability in Phase I, we will proceed to Phase II.

Phase II: Evaluation on the bioavailability of ranitidine in male and female rats in the presence of Cremophor RH 40, Poloxamer 188, Tween 80 and Span 20 at a series of doses utilized in the commercial formulations. The concentrations were shown in Table 5.1.

Table 5.1 Concentrations and applications of excipients in pharmaceutical formulation.

Excipients	Concentration (%)	Use
Cremophor RH 40	0.1	used to solubilise ethereal oils, perfume compositions, vitamins and hydrophobic active substances in aqueous- alcoholic and purely aqueous solutions.
	1	
	5	
	10	
	20	
Poloxamer 188	0.1	fat emulsifier; flavor solubilizer;
	1	spreading agent; wetting agent
	5	stabilizing agent; suppository agent;
	10	tablet coating; tablet excipient
	20	gelling agent
Tween 80	0.1	nonionic surfactant in ophthalmic preparations
	1	
	5	nonionic surfactant in lipophilic bases
	10	used in combination with plasticizing agents
	15	solubilizing agent in lipophilic bases; emollient in topical formulations; emulsifying agent in oil-in-water emulsions
Span 20	0.5	nonionic surfactant and solubilizing agent for insoluble, active constituents in lipophilic bases
	1	emulsifying agent used in combination with hydrophilic emulsifiers in oil-in-water emulsions and to increase the water-holding properties of ointments; solubilizing agent for poorly soluble, active constituents in lipophilic bases
	5	
	10	
	15	emulsifying agents used alone in water-in-oil emulsions

Phase I

5.2 MATERIALS AND METHODS

5.2.1 Materials

Ranitidine hydrochloride, glacial acetic acid and sodium acetate trihydrate were obtained from Sigma Aldrich (Dorset, UK). Span 20 and Tween 80 were purchased from Fluka (Dietikon, Switzerland). Poloxamer 188 and Cremophor RH 40 were from Agenda (Bradford, UK) and BASF (Cheadle, Germany), respectively. Water and acetonitrile were purchased from Fisher Scientific (Loughborough, UK), and were of HPLC grade.

5.2.2 Animals

Animals used for the experiments were male and female Wistar rats (8 weeks old), purchased from Harlan UK Ltd (Oxfordshire, UK). Male and female Wistar rats weighed approximately 250g and 200g, respectively.

All the animal work was conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act, 1986. The rats were housed at room temperature (25℃) and in a light-dark cycle of 12h. They were caged in groups of six, allowed to move freely and provided with food and water before the experiment. The day before the experiment, they were fasted overnight and individually housed in metabolic cages.

5.2.3 Effect of excipients on the intestinal transport of ranitidine

5.2.3.1 Tissue preparation

On the day of the experiment, rats were sacrificed with a CO_2 euthanasia chamber (Schedule 1 method) and the intestine was rapidly removed. The jejunum (10cm from the ligament of Treitz) was cut from the whole intestine, washed with cold KBR solution and put into beakers with KBR solution on ice. The tissue was then allowed to rest for approximately 20 minutes for lowing the tissue temperature to minimize tissue damage during preparation. About 2-3 cm long pieces from the proximal part of the jejunum were opened along their mesenteric border.

5.2.3.2 Ussing chamber set-up

When the preparation was finished, tissues were mounted in the vertical Ussing Chamber (Harvard Apparatus Inc., Holliston, MA, USA) as flat sheets on a 0.32cm^2 segment holder with needles to stabilize it. A 5mL KBR solution was added to each compartment of the Ussing Chamber and the solutions were gassed with an O_2/CO_2 (95%/5%) gas mixture. The chambers were screwed tight and the entire assembly was kept at 37℃.

To evaluate tissue integrity during experiments, tissue transepithelial electrical resistance (TEER) was measured using an EVOMX meter (World Precision Instruments Inc., WPI, Hertfordshire, UK). Any jejunum tissue presented a value of TEER lower than 40Ω•cm^2 at the beginning of experiment was regarded as poorly viable and excluded immediately. When TEER values decreased more than 15% from the value measured at the end of equilibration period, the tissue was considered not viable.

5.2.3.3 Transport study

After an equilibrium period of 20-30 minutes, the experiment was initiated by replacing the blank KBR solution with pre-warmed ranitidine solution in the absence and presence of pharmaceutical excipients in the donor compartment. The appropriate concentrations were shown in Table 5.2.

Table 5.2 Concentrations of excipients used in the *in vitro* studies.

Excipients	Mean Molecular Weights	Concentration (%)
Poloxamer 188	4600	5.5
Cremophor RH 40	2500	3
Tween 80	1310	1.6
Span 20	346	0.4

The permeation experiment lasted 3h and 100μL of receiver solution was taken for measuring the drug amount with HPLC method every 30min. An equal volume of heated blank KBR solution was added immediately after each sample was withdrawn.

5.2.3.4 Calculation

The P_{app} of each experiment, in cm/s, was computed using the equation followed:

$$Papp = \frac{Q}{C \cdot A \cdot t}$$

where Q (μmol) is the total amount of drug that permeated to the receiver compartment throughout the incubation time, C (μmol/mL) is the initial drug concentration in the donor side, A (cm^2) is the diffusion area of the Ussing Chamber, and t (s) is the time of experiment.

5.2.4 Influence of excipients on the bioavailability of ranitidine

Each rat was weighed on the day of the experiment and administered ranitidine solution in the absence and presence of pharmaceutical excipients using an oral gavage syringe. Doses were showed in Table 5.3.

Table 5.3 Concentrations of excipients used in the *in vivo* studies.

Excipients	Mean Molecular Weights	Concentration
Poloxamer 188	4600	295
Cremophor RH 40	2500	160
Tween 80	1310	84
Span 20	346	22

Then, approximately 250μL-300μL of blood was collected from the tail vein of rats into anticoagulant centrifuge tubes (BD Microtainer® K2E Becton, Dickinson and Company, USA) at 0.5, 1.25, 2, 3, 4 and 6h. At 8h post-administration, the rats were sacrificed with a CO_2 euthanasia chamber (Schedule 1 method) and about 1mL of blood was taken via cardiac puncture immediately. All blood samples were centrifuged at 10000rpm (930g) for 10 min on a Centrifuge 5804R (Eppendorf AG, 22331 Hamburg, Germany) within 8h of sampling. 50μL of the supernatant was collected and placed into a 1.5mL Eppendorf tube, the same volume of acetonitrile was added to precipitate the plasma proteins. After 1min of vortex-mixing, 100μL HPLC grade water was added to the mixture and after subsequent vortex-mixing, the samples were centrifuged at 4℃ for 10 min at 10000rpm. The supernatant was collected and kept at 4℃ until analysis.

5.2.5 Methods of analysis

The samples were stored in freezer at 4℃ until use and allowed to thaw at room temperature before processing. The sample was subjected to HPLC-UV analysis using a previously validated method. The column used was a 5μm Luna SCX (Phenomenex, UK); the mobile phase was a mixture of 20:80 (acetonitrile):(0.1M sodium acetate pH=5.0) with a flow rate of 2ml/min and 40μL of injection volume.

5.2.6 Pharmacokinetic analysis

Pharmacokinetic parameters, involving C_{max}, t_{max}, AUC_{0-480}, AUC_{∞}, CL, Vd and $t_{1/2}$ were calculated by non-compartmental analyses using a free Microsoft Excel add-in, "PKSolver." (Zhang et al., 2010).

5.2.7 Detection of P-gp protein level by Western blotting

5.2.7.1 Animal treatment

On the day of the experiment, each rat was weighed and orally administered with/without the pharmaceutical excipient solutions (doses were the same as what used in Table 5.3). 15min later, rats were sacrificed in a CO_2 euthanasia chamber and the intestine was rapidly removed. The jejunal intestinal pieces were prepared as described in Session 5.5.3.1.

5.2.7.2 Sample preparation

The mucosa (about 60mg) were cut into some small pieces and homogenized in 3mL lysis buffer at 10,00rpm 20s with a T18 digital ULTRA-TURRAX® (IKA). The tissue homogenates were incubated at 4°C for 2h and centrifuged at 10,000rpm for 10min. The supernatants were collected and stored in aliquots at -20°C until use.

5.2.7.3 Protein analysis

Protein concentration of each homogenate was determined with the Pierce™ BCA Assay Protein kit (ThermoFisher, Loughborough, UK) according to the manufacturer's instructions.

For gel electrophoresis, 25µg of total protein of each sample was suspended in LDS sample loading buffer (Invitrogen, Carlsbad, CA) and denatured for 10 min at 70°C. As a molecular weight marker, 5µL Sharp Pre-Stained protein standard (Invitrogen) was loaded on each gel. Samples were separated by electrophoresis in a NuPAGE™ NovexTM 4–12% Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane with XCell SureLock™ Mini-Cell Electrophoresis System (Invitrogen) according to the manufacturer's instructions. Membranes were blocked with 3% bovine serum albumin (BSA) in TBS-T and incubated for 1h at room temperature. For detection of P-glycoprotein and reference protein, blots were incubated 1h at room temperature with the respective primary antibodies diluted in 3% BSA in TBS-T: mouse monoclonal anti-Pgp (C-219 3:200; Enzo Life Science, Exeter, UK) and anti-β-actin mouse monoclonal antibody (1:2000; Sigma-Aldrich, Poole, UK). Detection of bound antibodies was done with affinity-purified rabbit anti-mouse IgG coupled to peroxidase (secondary antibody; Sigma) diluted 1:5000 in 3% BSA in TBS-T.

After 1h of incubation with secondary antibody conjugated with horseradish peroxidase, protein bands were visualized by chemiluminescence detection method with Pierce™ ECL Western Blotting Substrate (ThermoFisher) and blots were photographed with a ChemiDoc XRS camera (Bio-Rad). Detection for bands of P-gp and reference protein weas performed with the Image Lab™ software (Bio-Rad). For a calculation of the relative P-gp expression in selected groups, the reference protein was individually set to 1 and the intensity of P-gp was set relative to it.

5.2.8 Measurement of P-gp mRNA expression by real-time reverse-transcription polymerase chain reaction

5.2.8.1 RNA isolation

Rats were pre-treated as described in Section 7.2.7.1. All pieces of mucosal tissue were cut and kept in RNA later® Stabilization Solution (Thermofisher). Total RNA of each piece was isolated and purified with PureLink® RNA Mini Kit (Thermofisher) according to the manufacturer's instructions. RNA concentration was measured with Nanodrop 2000 (Thermofisher).

5.2.8.2 RNA level analysis

1mg total RNA of each sample was reverse transcribed using the iScriptTM cDNA Sythesis Kit (Bio-Rad). To quantify the amount of *mdr1a and mdr1b* mRNA, real-time PCR was performed on the 7500 Real Time PCR System (Applied Biosystems, Thermofisher) using the method described by MacLean (MacLean et al., 2008). Briefly, 50μL PCR reaction contained 25μL of PowerUp^{TM} SYBR Green PCR Master Mix (Thermofisher), 500nM each of forward and reverse primers, and 1μg of cDNA. Anti-beta actin was used for normalization and amplified of 1μg cDNA, respectively. Real-time PCR was carried out in 96 well PCR plates (Thermofisher). The amplification program for all genes consisted of one pre-incubation cycle at 95°C with a 10min hold, followed by 45 amplification cycles with denaturation at 95°C with a 10s hold, an annealing temperature of 50°C with a 10s hold and an extension at 72°C with a 10s hold. Amplification was followed by a melting curve analysis which ran for one cycle with denaturation at 95°C with a 1s hold, annealing at 65°C with a 15s hold and melting at 95°C with a 1s hold. Water was included as a negative control in each run to access specificity of primers and possible contaminations. Primers (shown in Table 4.5 in Chapter 4) were designed by primer-BLAST searching using publicly available sequence information of the GeneBank of the National Center for Biotechnology Information (NCBI) and

purchased from Eurofins (Eurofins Genomics, Germany).

5.2.8.3 Data analysis

Relative expression of *mdr1a* and *mdr1b* mRNA in different intestinal divisions were calculated using 7500 software (version 2.0.6, Thermofisher). The average of the Ct values for tested genes (*mdr1*a and *mdr1b*) and the internal control (anti-beta actin) were taken, and then the differences between Ct values for target genes and internal control (ΔCt) were calculated for all the experimental samples. ΔΔCt was used to present the relative quantification of tested samples according to the control.

5.2.9 Statistical analysis

The experiments were performed at least six times and data were expressed as mean±standard deviation (SD). Significant differences between groups were analyzed by one-way ANOVA, followed by a Tukey post-hoc analysis with a 95 % confidence interval using IBM SPSS Statistics 19 (SPSS Inc., Illinois, USA).

5.3 RESULTS AND DISCUSSION

The solubilizing agents evaluated in this study were by no means inert regarding relevant interactions with the function of P-gp, thereby altering the absorption of P-gp-mediated drug ranitidine (shown in Figure 5.2-5.6).

In detail, the presence of Poloxamer 188 increased the bioavailability and uptake transport of ranitidine by 10% and 46% in male rats respectively, whilst there were no changes demonstrated in the female ($p>0.05$). In addition, Poloxamer 188 reduced the P-gp protein and the corresponding *mdr1a* mRNA expression in male rats only. *Mdr1b* mRNA, however, was relatively evenly expressed and effected compared with the *mdr1a* isoform.

Correspondingly, Tween 80 was a potent excipient to modulate the activity and expression of efflux transporter P-gp, in that enhancing the absorption of ranitidine in male rats but not in female ones. In the male rats, the bioavailability and uptake transport of ranitidine increased by 29% and 24% respectively with Tween 80. Consonantly, there were 0.6-fold decreases in the P-gp protein abundance in the presence of Poloxamer 188.

Cremophor RH 40 also showed a sex-related impact on the absorption of ranitidine via the inhibition on the P-gp protein and gene expression. The bioavailability and uptake of ranitidine in male rats was increased by 30% and 33% respectively, with a 0.6-fold decrease in the P-gp protein content and *mdr1a* mRNA expression in the presence of Cremophor RH 40. Interestingly, with regard to the female rats, Cremophor RH 40 decreased the intestinal uptake of ranitidine by 25% compared to the control, however, no impacts were found in the bioavailability and P-gp expression ($p>0.05$).

Span 20, on the contrary, reduced the P-gp protein level by 41% and 59%, thereby increasing the uptake of P-gp substrate ranitidine by 21% and 23% in both male and female rats. Additionally, the presence of Span 20 enhanced the bioavailability of ranitidine in 37% and 20% in male and female rats respectively ($p<0.05$), which led to no sex differences in the effect of Span 20 on either ranitidine absorption or P-gp expression.

We could clearly demonstrate that Cremophor RH 40, Poloxamer 188, Tween 80 and Span 20 are able to significantly modulate the function and expression of efflux transporter P-gp. However, Span 20 is the only solubilizing agent studied here showed its interaction with membrane transporter to alter the drug bioavailability in both sexes, whilst the others exhibit their impacts only in male rats but not the female ones. Since these excipients are frequently used in the formulation for poor-soluble drugs, and P-gp is shown to be a major efflux transporter in the absorption and disposition of many drugs, sex-specific influences of these excipients on the pharmacokinetic of P-gp-mediated drugs were expected.

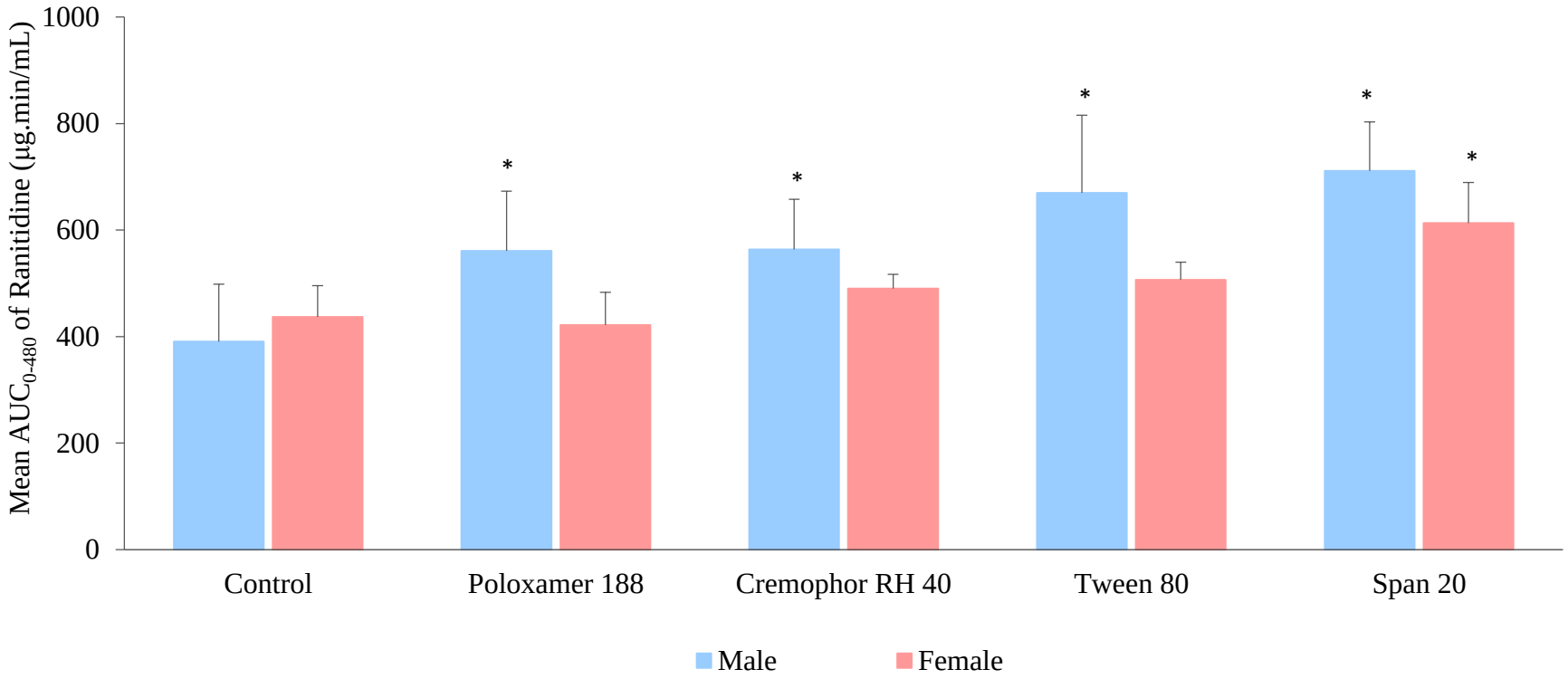


Figure 5.2 Mean AUC_{0-480} of ranitidine in the presence and absence of excipients in male and female Wistar rats (Mean ± S.D., n=6).
* Values are statistically different between the control and tested groups at $p<0.05$.

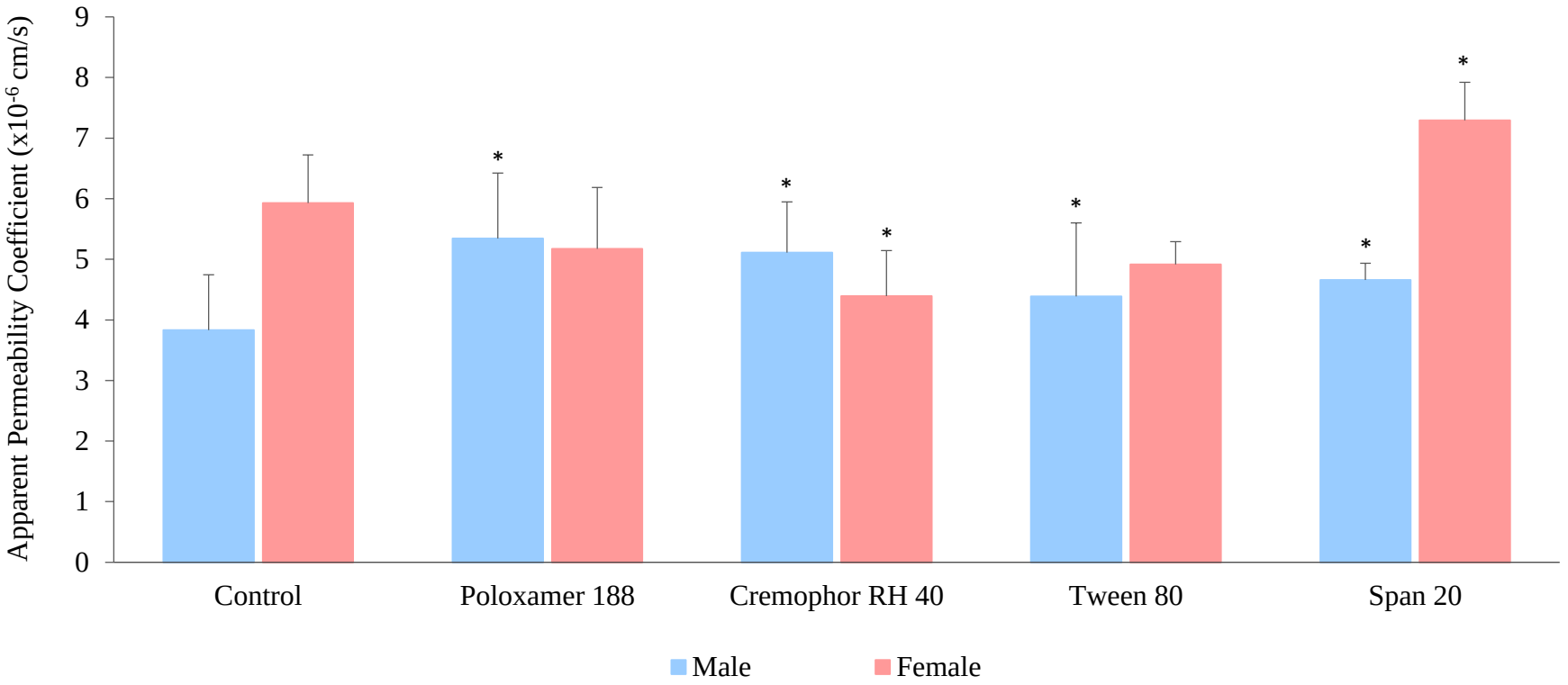


Figure 5.3 Permeability of ranitidine in the absence and presence of excipients in male and female Wistar rats (Mean ± S.D., n=6).
* Values are statistically different between the control and PEG group at $p<0.05$.

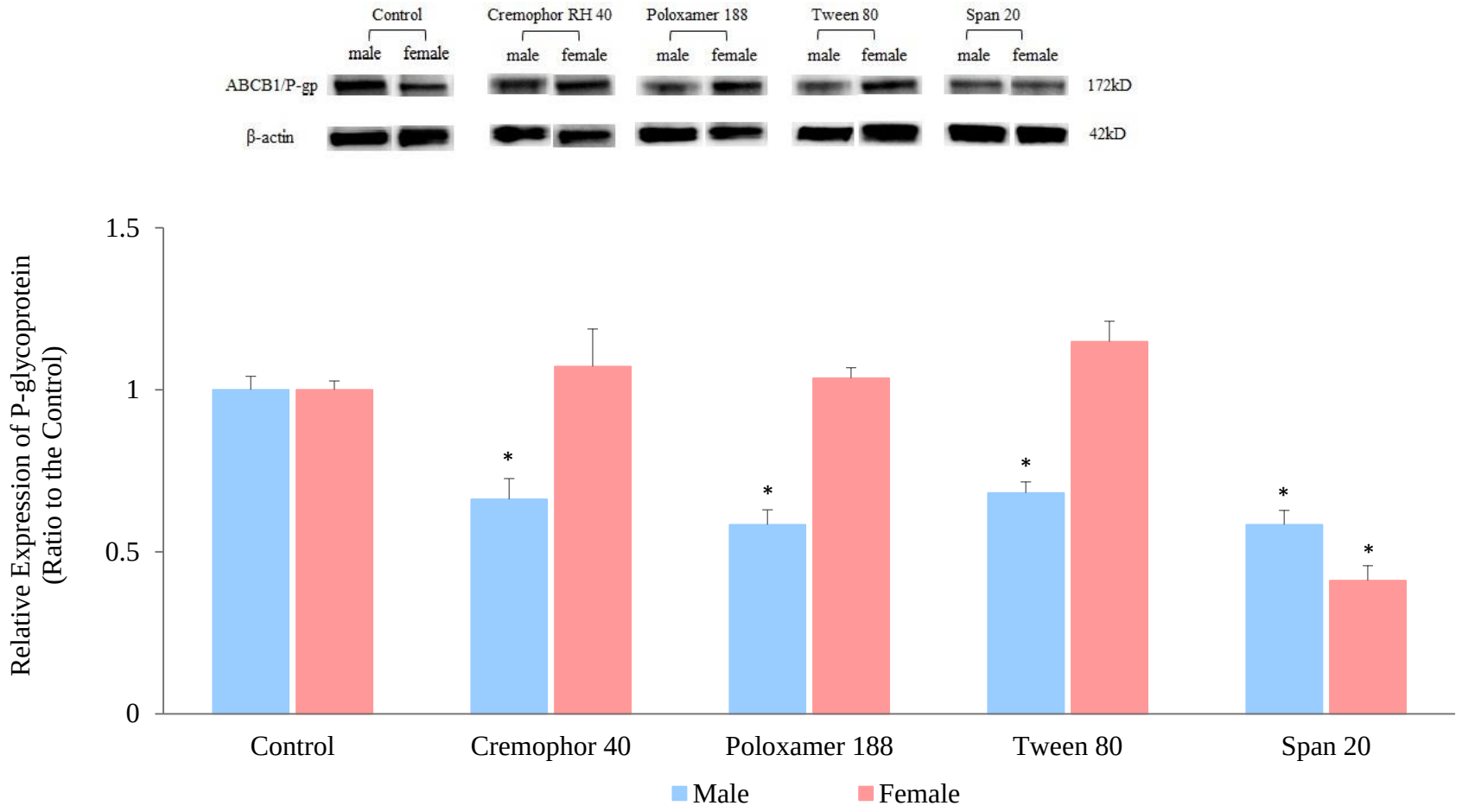


Figure 5.4 Fold changes in P-gp protein expression in jejunum of rats after 1h administration with excipients.
* Values are statistically different between the control and PEG groups at $p<0.05$.

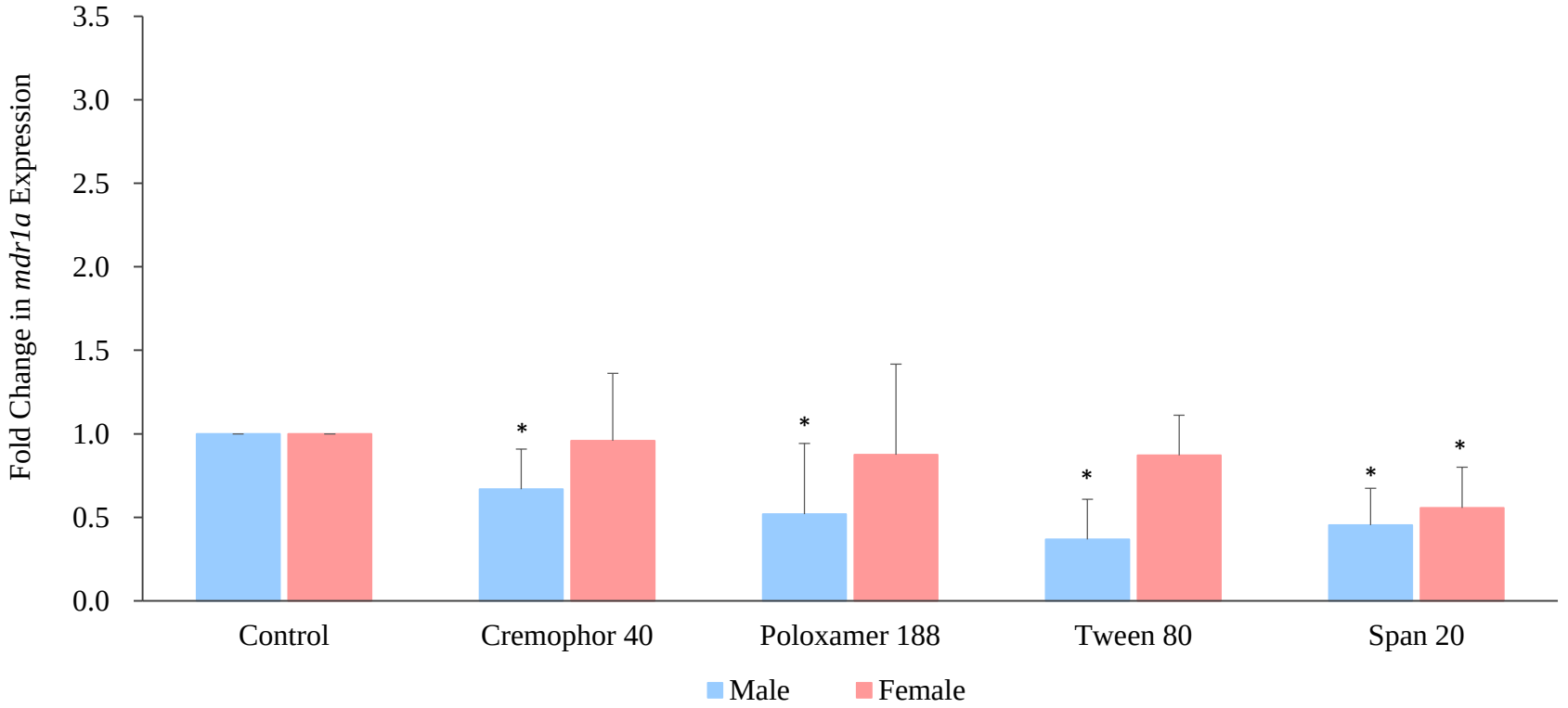


Figure 5.5 Fold changes in *mdr1a* expression in jejunum of rats after 1h administration with excipients.
* Values are statistically different between the control and PEG groups at $p<0.05$.

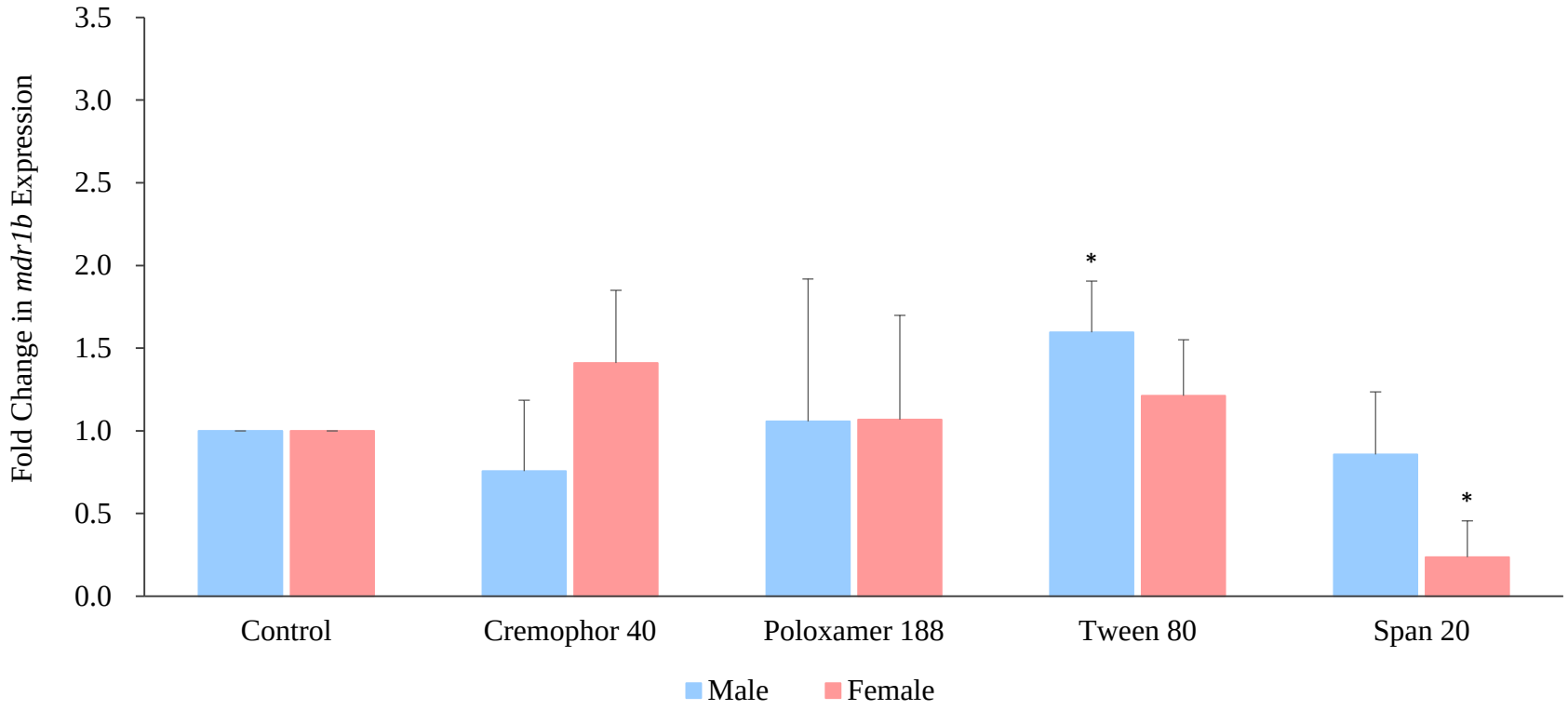


Figure 5.6 Fold changes in *mdr1b* expression in duodenum of rats after 1h administration with excipients.
* Values are statistically different between the control and PEG groups at $p<0.05$.

5.3.1 Cremophor RH40

Cremophor RH40 (polyoxyl 40 hydrogenated castor oil) is used as a solubilizer for various hydrophobic drug moieties. It was suggested to be a useful excipient particularly for enhancing bioavailability of P-gp substrates, considering its effects on efflux transporter, P-gp. However, interaction of Cremophor RH 40 with P-gp was sex-dependent in our study, where Cremophor RH40 inhibited the function of P-gp in male rats but not in females.

This finding in the males was in line with the previously published data. In details, Cremophor RH40 was found to increase the absorption of a P-gp substrate digoxin resulting in its increased bioavailability by up to 22% in a clinical trial on male individuals, and this *in vivo* human data supports the validity of *in vitro* observations on P-gp (Tayrouz et al., 2003). Taking this inhibitory effect on P-gp, Cremophor RH40 has also been used as a surfactant to increase bioavailability of tacrolimus (a poorly water soluble P-gp substrate) in male rats by formulating self-microemulsifying drug delivery system (SMEDDS) (Wei et al., 2005). After all, sex-related effect of Cremophor RH 40 on the P-gp function observed in this study highlights the safety on the application of Cremophor RH 40 as solubility and permeability enhancer in the oral drug formulations, if they are P-gp substrates.

5.3.2 Poloxamer 188 (Pluronics F68)

Poloxamers (poly (ethylene oxide) – blockpoly (propylene oxide) – block – poly (ethylene oxide)), also known as Pluronic, were widely used as O/W type emulsifying agents, viscosity increasing excipients and for surfactant gels.

Pluronics P85 was found to inhibit both MRP-1 and MRP-2 in a study performed on MDCK cells resulting into increased intracellular accumulation of vinblastine and doxorubicin (MRP substrates) (Batrakova et al., 2003). Apart from MRPs, Pluronics P85 showed its inhibitory action on P-gp present on the Caco-2 cells (Batrakova et al., 1998).

Other Poloxamers, like Poloxamer 188 (Pluronics F68), also displayed its impact on the P-gp-mediated transport of celiprolol and CYP3A4-mediated formation of midazolam metabolite 1'-hydroxymidazolam in Caco-2 cell monolayer, indicating that it is a potent in vitro inhibitor of both P-gp and CYP3A4 (Huang et al., 2008). Poloxamers are incorporated into membranes followed by subsequent translocation into the cells and affecting various cellular functions, such as mitochondrial respiration, ATP synthesis, activity of drug efflux transporters, apoptotic signal transduction and gene expression. This enhances drug transport across the blood brain and intestinal barriers, and causes transcriptional activation of gene expression both *in vitro* and *in vivo*. (Kabanov et al., 2002, Batrakova and Kabanov, 2008). Frustratingly, different activity of Poloxamers were observed between males and females in this study, resulting in differential influences on the function of P-gp and absorption of a P-gp substrate ranitidine in male and female rats. This information emphasizes sex differences in the bioavailability of drugs with flooding active pharmaceutical excipients.

5.3.3 Tween 80

Another widely-used nonionic surfactant, Tween 80 (polyoxyethylene 20 sorbitan monooleate), was also pharmacologically-active. Clearly, it was found to increase apical-to-basolateral permeability and decrease basolateral-to-apical permeability of Rhodamine 123 (a P-gp substrate) in Caco-2 cell monolayers at concentrations ranging from 0.01mM to 1mM in a dose-dependent manner (Rege et al., 2002). In another study, P-gp inhibition by Tween 80 was found for a model peptide drug (Acf $(NMef)_2NH_2$) in Caco-2 cell lines, where basolateral-to-apical permeability of the drug was decreased mostly with Tween 80 whose CMC was below 50 μM, indicating that monomers of this surfactant were responsible for its activity and not its micellar forms (Nerurkar et al., 1997). Subsequently, Tween 80 was reported to inhibit the activity of MRP2 slightly (Hanke et al., 2010). Recently, Tween 80 at concentrations below 0.01% (w/v) was able to decrease the expression of P-gp, resulting in an enhanced uptake of P-gp-mediated Rhodamine 123 (Hodaee et al., 2013). Although no *in vivo* and clinical studies have been carried out to describe the effects of Tween 80 on the function of P-gp, *in vitro* results strongly prove its affects. Multiple effects like P-gp alteration and enhanced solubility render it a valuable excipient for bioavailability improvement of poor water soluble P-gp substrates. However, Tween 80 herein was not only proved its active interaction with efflux transporter P-gp, but also reported its sex-dependent manner in the impact on the P-gp function.

5.3.4 Span 20

Span 20 (sorbitan monolaurate) significantly increases the absorption of ranitidine via the reduction on the intestinal P-gp expression in both male and female rats in this study. This result was in an agreement with the published information that Span 20 showed an inhibitory effect on the efflux transporters P-gp and BCRP in Caco-2 cell monolayers (Yamagata et al., 2007).

Judging from our data, lipid-based solubilizing excipients Cremophor RH 40, Poloxamer 188, Tween 80 and Span 20 all exhibited their inhibitory influences on the activity and expression of efflux transporter P-gp. Some mechanisms by which excipients non-specifically inhibit P-gp activity have been proposed and carefully investigated. For example, surfactants have been shown to modulate P-gp activity through altered fluidity of the lipid membrane environment of P-gp, leading to a reduction of ATPase activity, as well as decreased the affinity of P-gp for ATP with depletion of intracellular ATP (Cornaire et al., 2004). On the other hand, the expression of P-gp was recently reported to be linked to a group of nuclear receptors, which have lipid ligands providing interplay between lipids and transcriptional control of P-gp (Lee et al., 2006b, Chawla et al., 2001, Bookout et al., 2006). Such as the nuclear receptor proteins retinoic acid receptor (RAR), farnesoid receptor (FXR), steroid-activated receptor (SXR) and pregnane receptor in rodents (PXR) (Denson et al., 2002, Freedman, 1999, Yoshikawa et al., 2002). Therefore, lipid excipients studied in this paper may interact with the receptor pathways, resulting in the observed decreases in P-gp expression, similar to a previously reported lipid agent Peceol® and Gelucire® 44/14 (Sachs-Barrable et al., 2007).

However, these excipients, excluding Span 20, altered the P-gp function only in male rats but not in females. Sex difference is rarely reported on the influence of excipients on the function of membrane transporters. According to the data in this study, the surprising sex-related effect of excipients on the drug bioavailability may be related to the chemical structure of excipients, considering Cremophor RH 40, Poloxamer 188 and Tween 80 are all polyethoxylated surfactants. Taking PEG 400 into account, excipients are found to bind to P-gp in a substrate-like manner. Specifically, the interaction between excipients and P-gp in the lipid membrane is due to transient hydrogen bond formation of EO groups with hydrogen bond donor groups of the transmembrane domains of P-gp (Li-Blatter et al., 2009). Thus, more polyethoxylated agents widely-used in the formulations need to be studied their impacts on the function of membrane transporters in both males and females in the future.

5.4 SUMMARY

In Phase I, the influence of four excipients (Cremophor RH 40, Poloxamer 188, Tween 80 and Span 20) which are commonly used as solubilizing agents in formulations were investigated on their effects on the absorption of P-gp-mediated drug ranitidine, and the reason behind these influences was also studied. We identified that Cremophor RH 40, Poloxamer 188 and Tween 80 were capable of reducing the activity and expression of intestinal P-gp. This resulted in the enhancement of ranitidine bioavailability in male rats but not in females. No sex differences were found in the case of Span 20. Span 20, however, is not an example of a polyethoxylated agents when compared with the former four excipients. The mechanism underlying the sex-related influence of excipients on transporters may be related to the chemical structure of excipients.

However, only one dose of each tested excipients was investigated. Considering the aforementioned influence of PEG 400 on drug bioavailability was not only sex-related but dose-dependent. More concentrations of these tested excipients will be studied in Phase II.

Phase II

5.5 MATERIALS AND METHODS

5.5.1 Materials

Ranitidine hydrochloride, glacial acetic acid and sodium acetate trihydrate were obtained from Sigma Aldrich (Dorset, UK). Span 20 and Tween 80 were purchased from Fluka (Dietikon, Switzerland). Poloxamer 188 and Cremophor RH 40 were from Agenda (Bradford, UK) and BASF (Cheadle, Germany), respectively. Water and acetonitrile were purchased from Fisher Scientific (Loughborough, UK), and were of HPLC grade.

5.5.2 Animals

All the animal work was approved by the UCL School of Pharmacy's ethical review committee and was conducted in accordance with the home office standards under the Animals (Scientific Procedures) Act, 1986. Healthy male and female, 8-13 week old Wistar rats (Harlan UK Ltd, Oxfordshire, UK) weighing 150-250 g were used for excised rat intestine. The rats were housed at controlled temperatures (25℃) and humidity (50-60%) with a constant light-dark cycle of 12h, were provided with food and water and were acclimatized for 7 days before being studied. One day before the experiments, the rats were fasted overnight and housed individually in the metabolic cages.

5.5.3 Pharmacokinetic study

On the day of the experiment, each rat was weighed and administered 50mg/kg ranitidine in the absence or presence of excipients (doses of excipients were shown in Table 5.1) by oral gavage. Subsequently, approximately 250µL-300µL of blood was collected from the tail vein of the rat into anticoagulant centrifuge tubes (BD Microtainer® K2E Becton, Dickinson and Company, USA) at 0.5, 1.25, 2, 3, 4 and 6h. At 8h post-administration, the rats were killed in a CO_2 euthanasia chamber and about 1mL of blood was taken by cardiac puncture.

5.5.4 Samples preparation

Blood samples were centrifuged at 10,000rpm for 10min and the supernatants (plasma samples) were collected into 1.5mL Eppendorf tubes. The samples were prepared using a reported method (Afonso-Pereira et al., 2016). Briefly, 50µL of the supernatant was placed into a 1.5mL Eppendorf tube and the same volume of acetonitrile was added to precipitate the plasma proteins. After 1min of vortex-mixing, 100µL HPLC grade water was added to the mixture, which was vortex-mixed again for 30s, and centrifuged at 4ºC for 10min at 10000rpm. The supernatant was collected and 40µL aqueous was analyzed by HPLC.

5.5.5 Chromatographic analysis

Chromatographic analysis was performed with a high performance liquid chromatography (HPLC) system (Agilent Technologies, 1260 Infinity) equipped with pump (model G1311C), an autosampler (model G1329B) and a diodearray UV detector (model G1314B).

The sample was subjected to HPLC-UV analysis using a previously validated method (Ashiru et al., 2007). The column used was a 5µm Luna SCX (Phenomenex, UK); the mobile phase was a mixture of 20:80 (acetonitrile):(0.1M sodium acetate pH=5.0) with a flow rate of 2mL/min.

5.5.6 Pharmacokinetic analysis

Pharmacokinetic parameters, (C_{max}, t_{max}, AUC_{0-480}, AUC_{∞}, CL, Vd and $t_{1/2}$) were calculated by non-compartmental analyses using a free Microsoft Excel add-in, "PKSolver." (Zhang et al., 2010).

5.5.7 Statistical analysis

All results are expressed as mean ± SD (n = 6). The control and test group data were analyzed by one-way ANOVA, followed by post-hoc Tukey analysis with a 95 % confidence interval using IBM SPSS Statistics 16 (SPSS Inc., Illinois, USA).

5.6 RESULTS AND DISCUSSION

The bioavailability of ranitidine in the absence of excipients presented by the cumulative area under the plasma concentration versus time curve (AUC_{0-480}) in male and female rats was 338±27μg.min/mL and 451±53μg.min/mL, respectively, which was consistent with the value reported in previous study(Afonso-Pereira et al., 2016), where the bioavailability of ranitidine was comparable in male and female rats.

The enhancement on ranitidine bioavailability with excipients was in a dose-dependent and sex-dependent manner. Also, the bioavailability of a drug is influenced by many factors, including the solubility of drug. The solubility of ranitidine was measured and was in agreement with literature values. In this study, different concentrations of these solubilizing agents (Cremophor RH 40, Poloxamer 188, Tween 80 and Span 20) had no statistically significant effect ($p>0.05$) on the solubility of ranitidine, thereby eliminating it as a reason for any observed increases in drug bioavailability.

5.6.1 Cremophor RH 40

Cremophor RH 40 ranging from 0.001% to 20% was usually used in oral formulations as a solubilizer for fat soluble vitamins, essential oils and other hydrophobic pharmaceuticals, and also proved to have inhibitory effect on membrane transporters to enhance the drug permeability. The presence of 0.1%, 1%, 5% and 10% Cremophor RH 40 increased the bioavailability of ranitidine in male rats by 55%, 69%, 33% and 12% respectively ($p<0.05$). Pronounced bioavailability enhancement was observed with 1% Cremophor RH 40 dose, a 69% increase over the control ($p<0.05$) (shown in Figure 5.7).

The low doses Cremophor results in male rats reflect the findings in the previous literatures. In a study, the effects of a range of low concentrations of Cremophor RH 40 (0.001% to 3%) as potential inhibitors of P-gp were tested with the use of digoxin transport in Caco-2 cells. The results showed the inhibitory influence of Cremophor RH 40 on P-gp enhanced with the increase of its concentration, with 100% blocking of P-gp by 3% Cremophor RH 40 (Wandel et al., 2003). Moreover, in a clinical study, 600mg Cremophor RH 40 (corresponding to the concentration of 3% in this study) was reported to increase the bioavailability of digoxin by 22% in twelve male individuals via P-gp inhibition and prolongation of the dissolution time (Tayrouz et al., 2003).

When it comes to the high doses of Cremophor RH 40, it was usually used as surfactants in the emulsion formulations. The enhancement on the absorption of a P-gp substrate daidzein in the presence of 20% Cremophor RH 40 and 10% PEGs in male rats were demonstrated without explaining the underlying reason (Shen et al., 2011). In contrast, our data clearly show that a high dose of Cremophor RH 40 (20%) has a reduced influence on the bioavailability of P-gp-mediated ranitidine in male rats, thereby suggesting the influence of high doses of Cremophor RH 40 on the uptake transport of ranitidine and/or the pronounced prolongation of the dissolution time.

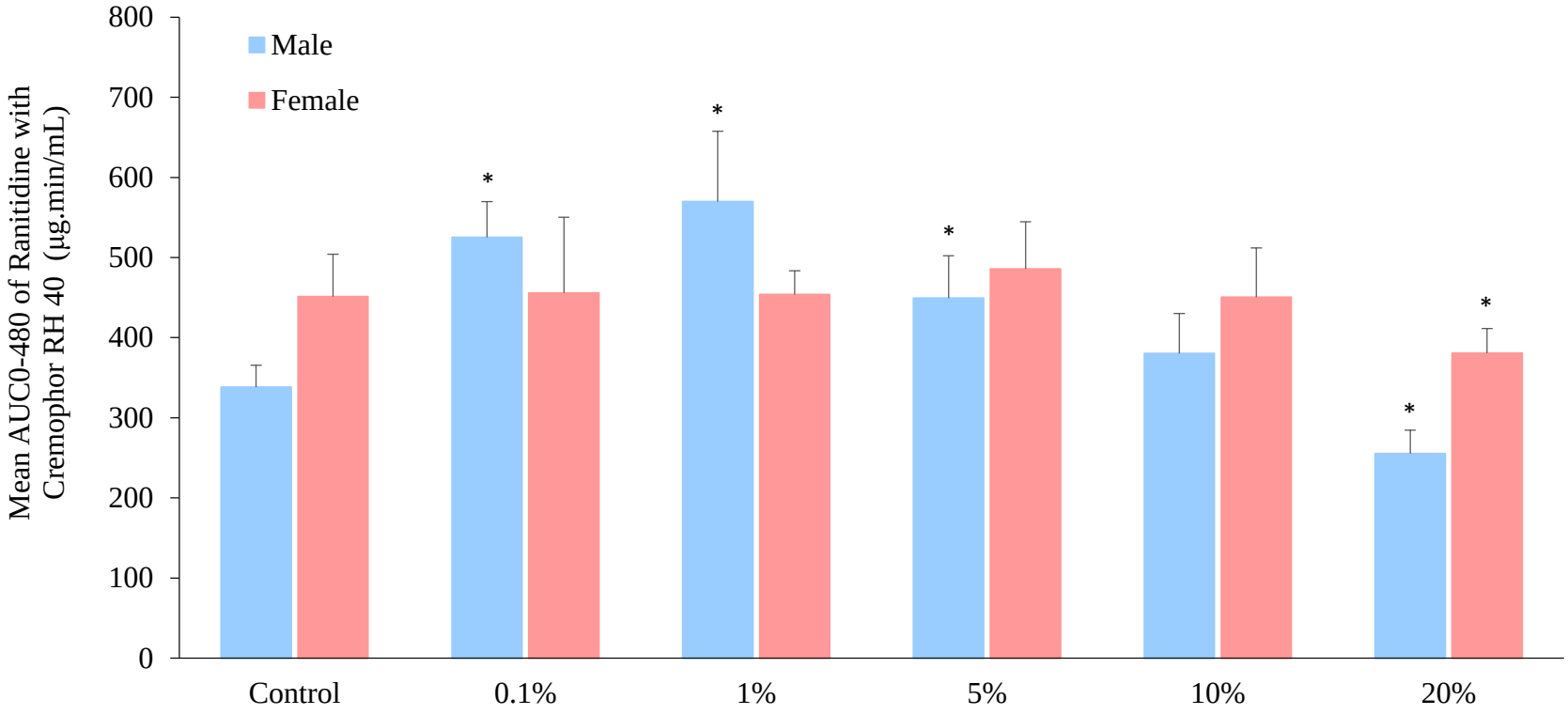


Figure 5.7 Mean $AUC_{0\text{-}480}$ of ranitidine in the presence and absence of Cremophor RH 40 ranging from 0.1% to 20% in male and female rats (Mean ± S.D., n=6). * Values are statistically different between the control and tested groups at $p<0.05$.

In contrast to the male rats, no significant changes were found in the bioavailability of ranitidine with 0.1%, 1%, 5% and 10% Cremophor RH 40 in female rats ($p>0.05$). Moreover, the ranitidine bioavailability with high concentration of Cremophor RH 40 (20%) in male and female rats was 255±29μg.min/mL and 381±31μg.min/mL respectively, corresponding to decreases by 25% and 16% compared with the control ($p<0.05$) (Figure 5.7).

The knowledge about the effect of Cremophor RH 40 on drug bioavailability in females was limited based on the previous reports. It however showed a clear statistical contrast to that seen in male rats ($p<0.05$) in our study. To be specific, in the females, the bioavailability of ranitidine was not influenced in the presence of Cremophor RH 40 in low concentrations, while the high dose of Cremophor RH 40 reduced the ranitidine absorption.

5.6.2 Poloxamer 188 (Pluronics F68)

Likewise, low concentrations of Poloxamer 188 (0.1%, 1%, 5% and 10%) enhanced the bioavailability of ranitidine in male rats, with the greatest enhancement by 55% found with 0.1% Poloxamer 188 compared to the control ($p<0.05$). In the female rats, only 10% Poloxamer 188 has a significant impact on the bioavailability of ranitidine with a 14% increase over the control ($p<0.05$). Additionally, alteration was not found in the ranitidine bioavailability in either male or female rats with high concentration Poloxamer (20%) (shown in Figure 5.8).

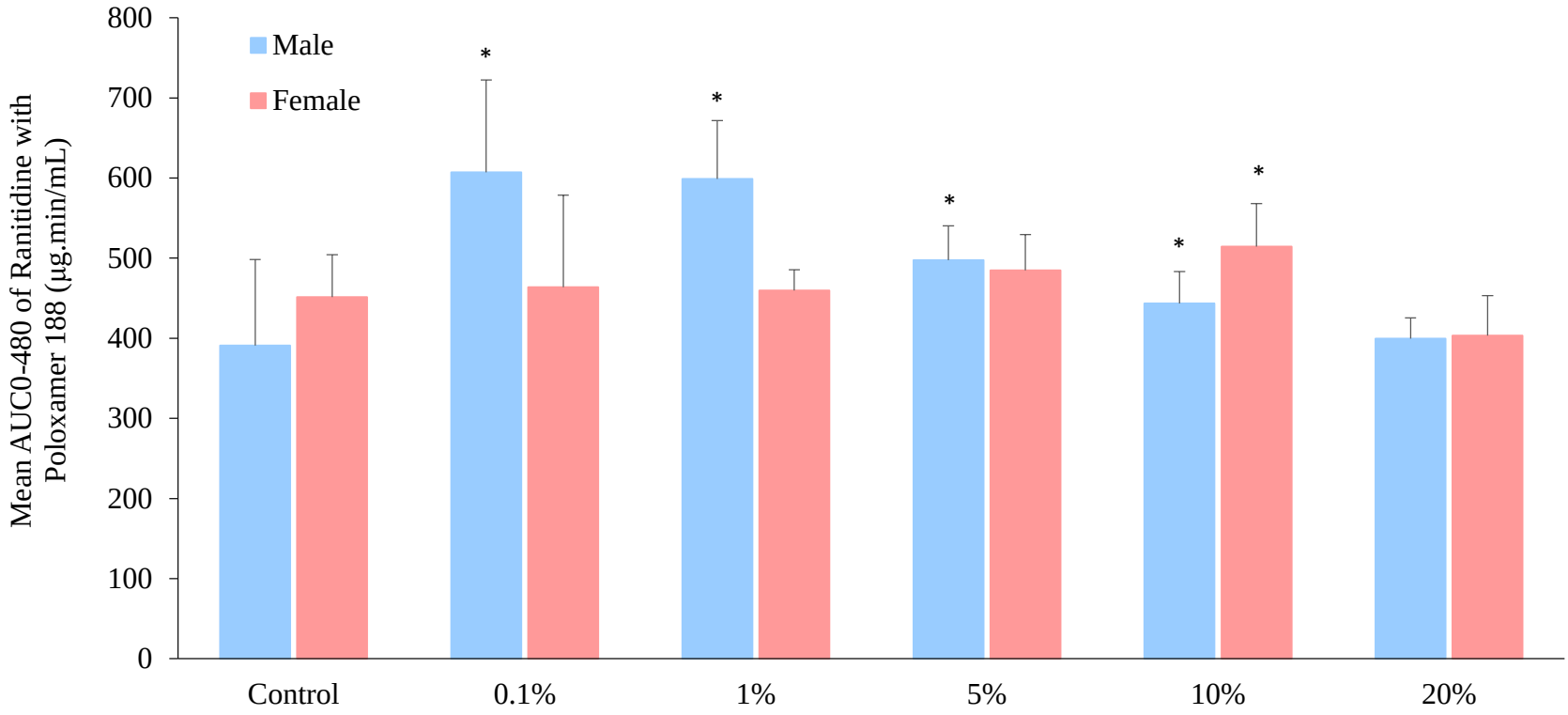


Figure 5.8 Mean $AUC_{0\text{-}480}$ of ranitidine in the presence and absence of Poloxamer 188 ranging from 0.1% to 20% in male and female rats (Mean ± S.D., n=6). * Values are statistically different between the control and tested groups at $p<0.05$.

Poloxamers (Pluronics) are nonionic polyoxyethylene-polyoxypropylene copolymer surfactants used primarily in formulations as emulsifying or solubilizing agents (Lee et al., 2003, Mata et al., 2005). Low doses of Poloxamers are usually used as emulsifying and solubilizing excipients used to maintain the clarity of elixirs and syrups whist high doses of Poloxamers are utilized as wetting agent in ointments, gelling agent, tablet binders and coatings.

Poloxamers mediate drug enhancement through efflux pump inhibition, often exhibited for blood-brain-barrier (BBB) drug delivery. The concentration-dependent efflux pump (P-gp) inhibitory effect of Pluronic 85 in brain microvessel endothelial cell monolayers was revealed. Furthermore it has been demonstrated that the efflux pump inhibitory effect of Poloxamers is reduced when its concentration reaches toward critical micelle concentration (CMC) (Miller et al., 1997).

Subsequently, an early study has successfully demonstrated larger gastrointestinal absorption of the aminoglycoside amikacin (P-gp substrate) following oral administration to mice in the presence of Poloxamer CRL-1605 (Jagannath et al., 1999). It also stands that dose-dependent influence of Poloxamers on the efflux transporters on the intestine. Although 0.8% Poloxamer 188 was reported not to inhibit intestinal P-gp to effect the transport of talinolol on male volunteers (Bogman et al., 2005), a significant ($p<0.05$) difference was later found in the transport of P-gp substrate diltiazem from the intestinal sacs of male rats pretreated with 0.25%, 0.5% and 1% Poloxamer 188 and control, indicating the inhibition of P-gp transporter and CYP3A enzyme respectively (Hafsa et al., 2015).

Apart from the inhibitory effect on membrane efflux transporters, Poloxamers can also be used to restore the membrane integrity attributed to its ability of interacting with the lipid bilayers and sealing the structurally damaged membranes. Based on this, several researchers have demonstrated the application of Poloxamer 188 as membrane sealing agent for therapeutic purposes due to its medical safety record (Lee et al., 1992, Padanilam et al., 1994, Merchant et al., 1998, Hannig et al., 2000, Frim et al., 2004).

5.6.3 Tween 80

It becomes apparent from Figure 5.9 that 0.1% Tween 80 was capable in increasing the bioavailability of ranitidine by 14% in male rats. The ranitidine AUC_{0-480} in the presence of 1% and 5% Tween 80 was 517±70μg.min/mL and 548±98μg.min/mL respectively, corresponding to 32% and 49% increases in bioavailability when compared with the control. On the contrary, low doses of Tween 80 have no influences on ranitidine bioavailability in female rats ($p>0.05$). However, ranitidine AUC_{0-480} in females could be enhanced with higher dose of Tween 80, for example, 15% Tween 80 almost doubled the bioavailability of ranitidine in females ($p<0.05$).

In pharmaceutical applications, Tween 80 is the most commonly used surfactant in FDA-approved parenteral products (Nema et al., 1997). It can be used as solubilizing agents for a variety of substances and also used to improve the chemical stability of solution formulation (Haque et al., 1999). Higher concentrations of Tween 80 were also used as plasticizing agents to reduce the melting temperature of drug molecules and the glass transition temperature of solid dispersion formulation (Ghebremeskel et al., 2007), while lower concentrations are used as emollients to increase the water holding capacity of the formulation in topical applications.

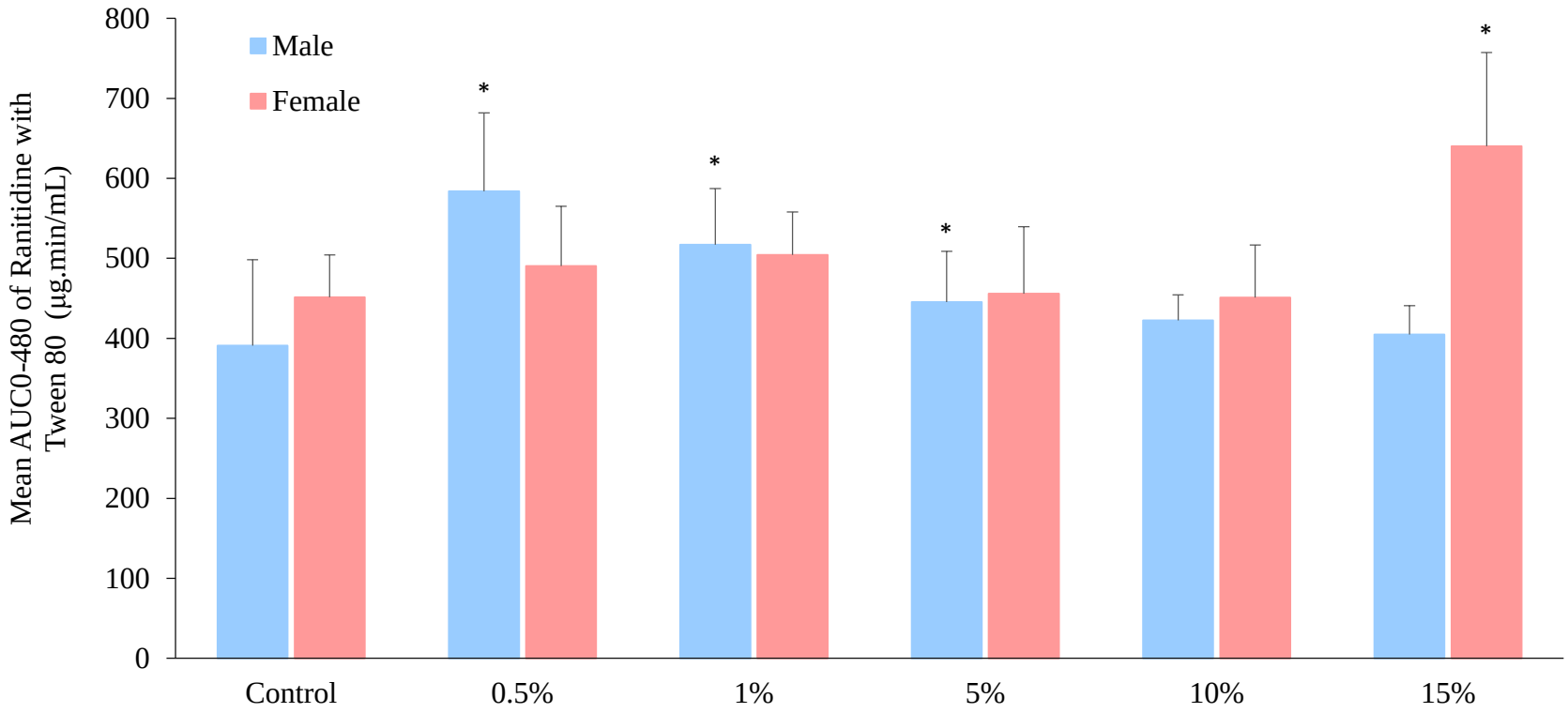


Figure 5.9 Mean $AUC_{0\text{-}480}$ of ranitidine in the presence and absence of Tween 80 ranging from 0.5% to 15% in male and female rats (Mean ± S.D., n=6). * Values are statistically different between the control and tested groups at $p<0.05$.

Tween 80 has been found to be useful in improving the oral bioavailability of drug molecules which are substrate for P-gp (Nerurkar et al., 1996). Firstly, Tween 80 was shown to enhance apical-to-basolateral permeability and decrease basolateral-to-apical permeability of a P-gp substrate Rhodamine 123 in Caco-2 cell monolayers at concentrations ranging from 0.01mM to 1mM in a dose-dependent manner due to Tween 80's inhibitory influence on efflux transporter P-gp (Rege et al., 2002). Secondly, the extent of P-gp inhibition on the cell line by Tween 80 was reported to depend logarithmically on excipient concentration, ranging from 0.5% to 3% (Sun et al., 2004). Thirdly, in the *in vivo* study, 10% Tween 80 showed a greater impact on the bioavailability of digoxin in a 61% increase in male rats ($p<0.05$), compared with 1% Tween which enhanced the drug bioavailability by only 30% (Zhang et al., 2003).

The advanced influence of Tween 80 at high doses on drug bioavailability was previously investigated as well: the application of 40% Tween 80 in a developed SEDDS (self-emulsifying drug delivery system) formulation for carvedilol increased its bioavailability in beagle dogs (males) up to 413% compared with commercially available tablets (Luode®). It indicated high concentrations of Tween 80 may induce the bioavailability of drugs via solubility enhancement and/or transporter modulation, however, the advantage of a new delivery system cannot be ignored (Wei et al., 2005).

Furthermore, a dose-dependent effect of Tween 80 on the function of P-gp could be explained by a clear inhibitory effect of Tween 80 on P-gp ATPase is observed at low concentrations which is lost at high concentrations. This is most likely due to a membrane loosening effect at high concentration of Tween 80 (Li-Blatter et al., 2009). However, it was not the same as what obtained in the female rats in study herein, where the bioavailability of ranitidine was increased in the presence of higher dose of Tween 80 but not low doses, suggesting sex differences in the membrane fluidity and/or response to excipients.

5.6.4 Span 20

Comparable data were shown in the case of Span 20 (shown in Figure 5.10). The presence of 15% Span 20 exhibited a 155% enhancement on the bioavailability of ranitidine in females ($p<0.05$), whereas lower doses of Span 20 did not alter the ranitidine $AUC_{0\text{-}480}$ ($p>0.05$). In the male rats, the bioavailability of ranitidine with 0.5%, 1%, 5%, 10% and 15% Span 20 was 562 ± 157μg.min/mL, 504 ± 51μg.min/mL, 390±87μg.min/mL, 433±30μg.min/mL and 437±47μg.min/mL, respectively. These correspond to 66%, 49%, 15%, 28% and 29% increase compared to the control treatment. Pronounced bioavailability enhancement was observed with 0.5% Span 20 which was a 66% increase over the control.

Span 20 are widely incorporated into cosmetics, food products and pharmaceutical formulations as lipophilic nonionic surfactants, mainly used as emulsifying agents and used at a low concentration of 0.1-3% in the preparation of a solubilizing agent for insoluble compounds in lipophilic bases (Fatouros et al., 2007).

Low doses of Span 20 (50μM and 100μM) were reported to increase the uptake of mitoxantrone in P-gp/MDCK-II and BCRP/MDCK-II cells significantly ($p<0.05$), suggesting that it could inhibit efflux transporters P-gp and BCRP. Also, it demonstrated that the Span 20 did not reduce the intercellular ATP in the cells, thereby indicating intercellular ATP levels played a minor role in the influence of Span 20 on membrane transporters (Yamagata et al., 2007).

Our data demonstrated Span 20 lower than 0.5% enhanced the bioavailability of ranitidine in males but not in females, while 1% Span 20 increased the drug bioavailability in both male and female rats, supporting the previous studies in some cases. In a similar manner as the high doses of Tween 80, Span 20 at high concentration (15%) enhanced the bioavailability of ranitidine in male and female rats with a five-time stronger response in females compared to the males.

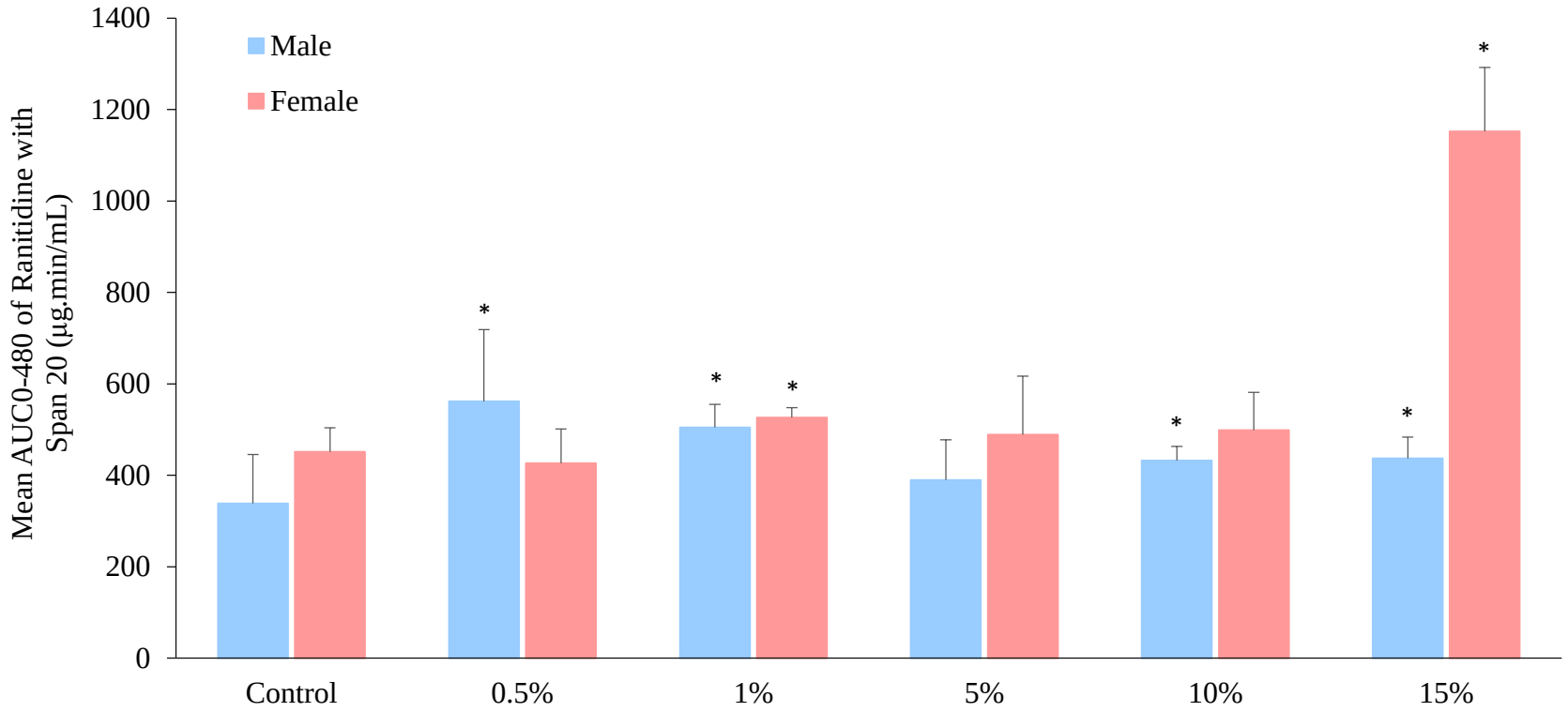


Figure 5.10 Mean $AUC_{0\text{-}480}$ of ranitidine in the presence and absence of Span 20 ranging from 0.5% to 15% in male and female rats (Mean ± S.D., n=6). * Values are statistically different between the control and tested groups at $p<0.05$.

5.7 CONCLUSION

Great progress has been made in the application of excipients in formulation development, not only for predetermined purposes but to also increase the drug bioavailability via effects on efflux transporters according to the work in this chapter. Briefly, commonly used excipients are emerging as a different class of P-gp inhibitors owing to advantages of being safe, not being absorbed from gut and pharmaceutically acceptable. However, some excipients showed different impacts on the function of transporters in males and females. For example, Cremophor RH 40, Poloxamer 188 and Tween 80 can modify the intestinal P-gp protein and mRNA expression to induce the bioavailability of ranitidine in male rats, but not in females.

These influences were also dose-dependent. Within the tested concentration range for all drug-excipient combinations, low doses of excipients caused increases in the bioavailability of ranitidine in male rats but not in females, while high doses of excipients enhanced the drug bioavailability in both male and female rats.

CHAPTER 6: Conclusion and future work

6.1 CONCLUSION

To clarify the reasoning behind the unexpected sex-associated influence of PEG 400 on the bioavailability of ranitidine in humans and rodents, we established that PEG 400 also had a sex-dependent impact on the absorption of ampicillin. This resulted to a marked increase in male rats but no alteration in females. Taking their absorptive mechanisms into consideration, we noticed both ranitidine and ampicillin are substrates for the efflux transporter P-gp, hereby estimating that this sex-based phenomenon only occur with P-gp-mediated drugs. Indeed, the presence of PEG 400 showed no effects on the bioavailability of metformin (a non-P-gp substrate) in neither males nor female rats, highly supporting our hypothesis.

Further investigations into the role of P-gp in the effect of PEG 400 on the bioavailability of P-gp substrates were conducted in the P-gp blocking animal models, who were pre-administrated with the P-gp inhibitor, CsA, for P-gp blockage. Interestingly, PEG 400 did not enhance the bioavailability of ranitidine or ampicillin when the P-gp of rats were pre-blocked. This data may suggest two aspects: firstly, PEG 400 has an inhibitory effect on efflux transporter P-gp to induce the absorption of drugs in male rats; secondly, sex differences may be observed in the interaction between PEG 400 and P-gp. CsA was then found to make a greater enhancement on the ranitidine bioavailability in male rats compared to the females, thus, a higher P-gp activity and/or larger P-gp expression may be obtained in males.

Subsequently, an *in vitro* model was established to characterize the influence of PEG 400 on intestinal transport of a P-gp substrate ranitidine in the intestine of male and female rats, with the aim to study the effect of PEG 400 on the P-gp activity and determine the *in vivo-in vitro* relationship of this sex-related phenomenon. The data showed that low concentrations of PEG 400 was capable of increasing the intestinal uptake of ranitidine in the jejunum, ileum and the colon in male rats,

although this was not observed in female rats when using the Ussing chamber system. However, no sex-based difference was found in the duodenum, suggesting that the sex-related phenomenon was region-related and dose-dependent. Moreover, the PEG 400-mediated modulation of P-gp activity accounted for changes in ranitidine intestinal transport and in marked differences between males and females. This was also observed in the CsA inhibitory effect which exhibited a higher P-gp activity in male rats when compared with females. Additionally, pre-treatment with CsA on rat tissues could eliminate this sex-based effect of PEG 400 on ranitidine intestinal transport. This further supports our hypothesis that in *in vivo* study, sex-related effect of PEG 400 on the drug bioavailability is mediated by the influence of PEG 400 on the efflux transporter P-gp. We speculate that sex-related differences in the variations in P-gp ATPase or ATP level in cells could cause sex differences in the interaction between CsA/PEG and P-gp. CsA was reported to alter the P-gp activity by inhibiting both the substrate stimulated and the basal P-gp ATPase, while PEG was found to influence the function of P-gp by producing mitochondrial toxicity and depleting the amount of intracellular ATP.

To better understand the sex differences from the influence of PEG 400 on the P-gp expression, we demonstrated that the presence of PEG 400 could alter the P-gp protein content and mRNA expression in the adult rat intestine from the jejunum to colon. This alteration was studied within 3hrs which then showed a sex-based difference. In the male rats, the intestinal P-gp protein abundance was reduced in the first hour and then increased slowly in the next two hours. On the contrary, P-gp protein levels were continuously decreased in female rats. The aforementioned data suggested that the intestinal membrane fluidity was more stable whilst the epithelial renewal in the intestinal epithelium was slower in females when compared with males. Also, sex differences were observed in the P-gp protein abundance in the absence of PEG 400, with a higher P-gp content in the jejunum, ileum and colon of male rats compared to females. In addition, the relative P-gp

protein content was highly correlated to the *mdr1a* expression, but not *mdr1b* gene expression, in both male and female rats.

Mechanistic investigation in the effects of more commonly-used solubilizing reagents Cremophor RH 40, Poloxamer 188, Tween 80 alongside Span 20 at one concentration on the bioavailability of ranitidine in male and female rodents (regarding their influence on the intestinal transport of ranitidine and P-gp expression) were conducted using the formerly developed methodologies. Surprisingly, Cremophor RH 40, Poloxamer 188 and Tween 80 showed a sex-dependent manner similar to PEG 400, in which an increase of bioavailability and permeation of ranitidine via the reduction on the P-gp activity and expression in male rats was identified, but not in female rats. On the contrary, the presence of Span 20 decreased the P-gp expression to induce the absorption of ranitidine in both male and female rats, which led to no sex differences. This finding illustrated that the mechanism underlying this sex-related influence of excipients on transporters may be related to the chemical structure of excipients as Span 20 is the only excipient which is not polyethoxylated among the tested excipients.

Extensive investigation on the influence of the bioavailability of ranitidine with the aforementioned “active” excipients was carried out at a wide range of concentrations. The results showed that Cremophor RH 40, Poloxamer 188, Tween 80 and Span 20 have a sex-associated and dose-dependent impact on the ranitidine absorption in rats. In detail, low concentrations of these four pharmaceutical excipients caused an increase in the bioavailability of ranitidine in male rats but not in females, whilst high concentrations of excipients enhanced the drug bioavailability in both male and female rats.

Overall, formerly regarded “inert” pharmaceutical excipients PEG 400, Cremophor RH 40, Poloxamer 188 and Tween 80 reduced the activity and expression of efflux transporter P-gp to enhance the intestinal absorption of P-gp-mediated drug ranitidine in male rats, but not in females. Based on these finding, we can provide suggestions. Firstly, pharmaceutical excipients used for specific predetermined purposes in formulation like solubility enhancement, taste masking and controlled release are also able to be enhance therapeutic efficacy of the drug by modulation of transporter activity and expression, therefore, two purposes can be simply achieved with the use of only one excipient in the formulation development. Secondly, the function of transporters are altered depending on the sex of the organism. This emphasizes the selection of excipients during formulation development to prevent different therapeutic efficacy and side effects between males and females.

6.2 FUTURE WORK

The findings in this study highlight the need of pharmaceutical companies or research groups to acknowledge that excipients selected during formulation development are capable to alter therapeutic efficacy and side effects between male and female participants. This work further raises several new aspects that could be investigated in the future.

Firstly, only five excipients were investigated of their potential sex-related influences on the efflux transporter P-gp. It is of vital importance to explore and identify more “active” excipients which may show sex-related effect on P-gp.

Lipophilic compounds and suspending agents reportedly interact with some membrane transporters. For instance, lipophilic compound cholesterol can influence the fluidity of the membrane (Baggetto and Testa-Parussini, 1990) and reduce the activity of efflux transporters such as P-gp and MRP (Ferte, 2000). It was first observed that the ATPase activity of MRP-1 was decreased by approximately 40% when the level of cholesterol in the proteoliposomes increased from 0 to 20–40%. On the other hand, it also demonstrated that cholesterol could improve the toxicity of some cytotoxic agents by affecting the ATPase activity of P-gp in the plasma membrane (Shu and Liu, 2007). Besides cholesterol, sodium taurocholate (NaTC) was used in orally administered liposomes to increase intestinal permeation of insulin, due to its enhancement on paracellular absorption by opening tight junctions (Johansson et al., 2002, Degim et al., 2004). Further experiments showed that NaTC could also increase the transcellular absorption of certain APIs by affecting P-gp mediated efflux (Ingels et al., 2004), when the effect of simulated intestinal fluid was investigated on drug permeability across Caco-2 monolayers (Ingels et al., 2002).

On other hand, Hydroxypropyl-β-cyclodextrin (HPCD) as one of the cellulose derivates, was reported to be a strong inhibitor of OATP1B3, OATP1A2 and OATP1B1 (Yan et al., 2008, Frijlink et al., 1991). Furthermore, HPCD and other cyclodextrins are known to form stable complexes with cholesterol, which can affect the fluidity of the membrane (Baggetto and Testa-Parussini, 1990) and decrease the activity of efflux transporters such as P-gp and MRP (Ferte, 2000).

It would be interesting to evaluate the influence of these active excipients on the bioavailability of ranitidine in male and female rats, following investigation on the reason behind the phenomenon if sex-related differences are shown.

Secondly, since an increasing number of pharmacologically active excipients were found to interact with various efflux transporters, uptake transporters and enzymes, sex differences in the interaction between these excipients and transporters need to be acknowledged.

Thirdly, the tested excipients were prepared with the model drugs in a water solution. It would be appropriate to explore if the sex-associated effect of excipients on the P-gp substrates still exist when excipients are co-formulated with P-gp-mediated drugs.

Taking Cremophor RH 40 as an example, it has been widely used as a surfactant to increase the solubility of poorly water soluble drugs in self-microemulsifying drug delivery system (SMEDDS). It would be of worth to establish a new formulation for poor-soluble drugs which are modified by P-gp using SMEDDS. Consequently, an investigation into its impact on the bioavailability of these drugs in male and female rats could be conducted.

Fourthly, novel formulations can be developed to enhance the bioavailability of drugs, and avoid the sex differences, based on our findings in this study.

Fast-releasing dosage forms would be an optimal choice in the oral formulation development of P-gp substrates, owing to lower P-gp content in the proximal small intestine disregarding both species and sex of the subjects. In addition, inclusion of "active" pharmaceutical excipients, such as PEG 400, are likely to benefit oral formulations containing P-gp substrates that are BCS Class IV drugs (poor solubility and poor permeability) as PEG 400 could both improve the solubility and permeability of co-formulated drugs.

Taking these two ideas into consideration, a proximal small intestine targeted formulation could be developed for BCS Class IV drugs with the application of active excipients. For example, testicular cancer is one of the cancers known to only affect men. Vinblastine is a typical chemotherapy medication to treat this cancer which is a BCS Class IV drug mediated by P-gp. Therefore, a fast-releasing oral formulation could be established for vinblastine. The addition of PEG 400, Cremophor RH 40, Poloxamer 188 or Tween 80 in the formulation would be beneficial to increase its solubility and permeability.

Last but not least, it would be useful to test the influence of other excipients on drug bioavailability in human, since all the studies in this thesis were conducted in rats.

Publications

Mai Y, Afonso-Pereira F, Murdan S, Basit AW. 2017. Excipient-mediated alteration in drug bioavailability in the rat depends on the sex of the animal. Eur J Pharm Sci. 12, 107: 249-255.

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